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PAPILLOMAVIRUS HUMANO: ESTUDO DE POLIMORFISMOS EM GENES DO INFLAMASSOMA E CITOCINAS

SÉRGIO FERREIRA DE LIMA JÚNIOR

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Tese apresentada ao Programa de Pós-Graduação em Biologia Aplicada à Saúde – Universidade Federal de Pernambuco, como requisito final para a obtenção do grau de Doutor em Biologia Aplicada à Saúde.

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RESUMO

O câncer cervical é o terceiro mais comum no mundial. Entretanto, apenas um pequeno número das infecções causam lesões ou evoluem ao que câncer, indicando a ação de outros agentes. SNPs em genes de inflamassomas e citocinas tem sido associado com respostas a infecções virais e ao desenvolvimento de neoplasias. O estudo teve por objetivo investigar relação entre SNPs de genes do inflamassoma e citocinas com a infecção pelo HPV e com a progressão da neoplasia intraepitelial cervical em pacientes da Região Metropolitana do Recife. Os SNPs foram acessados por PCR-SSP, PCR-RFLP, sondas Taqman ou sequenciamento de DNA. Observou-se que o polimorfismo do TNF-α rs1800629 foi associado com um maior risco de infecção por HPV [p= 0.0008]; NLRP1 rs11651270 desempenhou papel de proteção contra a persistência e/ou oncogênese do HPV [p= 0.0003]; *NLRP3* rs10754558 e *IL-18* rs1834481 [p=0.0008 e p= 0.018, respectivamente] possuiam efeito benéfico contra a persistência do HPV; *NLRP3* rs10754558, *IL-18* rs1946518 e rs2227307 [p= 0.0004 e p= 0.0043, respectivamente] estão significativamente associados com menor risco de infecção por HPV de alto risco; enquanto os polimorfismos *IL-1* rs1143634 e rs1143643 [p= 0.0001 e p= 0.04, respectivamente] estão associados à proteção pela infecção por HPV, sendo o rs1143634 associado à progressão de lesões cervicais uma vez que o HPV tenha se instalado [p= 0.0001]. Estes resultados ampliam o entendimento da relação entre polimorfismos nos genes dos inflamassomas e citocinas no risco de infecção por HPV e no desenvolvimento de lesões precursoras do câncer cervical.

Palavras Chave: Inflamassoma. NIC. SNP. Câncer Cervical. Citocinas.

ABSTRACT

Cervical cancer is the third most common in women worldwide. However, only a small number of infections cause injury or evolve to cancer, indicating the action of other agents. Inflamassomas SNPs in genes and cytokines has been associated with responses to viral infections and cancer development. The study aimed to investigate the relationship between SNPs inflammasome genes and cytokines with HPV infection and progression of cervical intraepithelial neoplasia in patients in the Metropolitan Region of Recife - Pernambuco - Brazil. SNPs were accessed by PCR-SSP, PCR-RFLP, Taqman probes or DNA sequencing. Our study showed that the TNF-α polymorphism of rs1800629 was associated with an increased risk HPV infection [P = 0.0008]; NLRP1 rs11651270 played a protective role against the persistence and / or HPV oncogenesis [p = 0.0003]; NLRP3 rs10754558 rs1834481 and IL-18 [P = 0.0008 and p = 0.018, respectively] had a beneficial effect against HPV persistence; NLRP3 rs10754558, rs1946518, and IL-18 rs2227307 [p = 0.0004 and p = 0.0043, respectively] are significantly associated with a lower risk of high-risk HPV infection; while IL-1 polymorphism rs1143634 and rs1143643 [p = 0.0001 and p = 0.04, respectively] are associated with protection by HPV infection, and the rs1143634 associated with progression of cervical intraepithelial lesions since the HPV has settled [p = 0.0001]. These results extend the understanding of the relationship beetwhen polymorphisms on inflammasome and cytokines genes in the risk of HPV infection and the development of precursor lesions of cervical cancer.

Keywords: Inflammasome. CIN. SNP. Cervical Cancer. Citokynes.

LISTA DE ABREVIATURAS E SIGLAS

APC – Célula Apresentadora de Antígeno

CARD – Domínio de Recrutamento Associado à Caspase

CCI – Câncer Cervical Invasivo

CD – Células Dendríticas

CSF – fatores estimuladores de colônia

CTL – Lectinas tipo C

DAMP – *Danger-Associated Molecular Pattern* – Padrões Moleculares Associados a Perigo

E – *Early* (precoce) – Região precoce do genoma do Papillomavírus Humano

HPV – Papillomavírus Humano

HR-HPV – *High Risk HPV* (HPV de Alto Risco)

HSV – Herpes simplex Virus – Vírus da Herpes simples

ICTV – *The International Committee on the Taxonomy of Viruses* – Comitê Internacional de Taxonomia Viral

IFN – Interferon

IL – Interleucina

L – *Late* (tardia) – Região tardia do genoma do Papillomavirus Humano

LCR – *Long Control Region* (Região Longa de Controle)

LR-HPV – *Low Risk HPV* (HPV de Baixo Risco)

MHC – Complexo de Histocompatibilidade Principal

NIC – Neoplasia Intraepitelial Cervical

NK – *Natural killers* (Células Matadoras Naturais)

NLR – *NOD-like Receptors* – Receptores tipo NOD

NLRP – Receptores tipo NOD Contendo Domínio Pirina

PAMPs – Padrões Moleculares Associados a Patógenos

PRR – Receptor de Reconhecimento Padrão

PV – Papillomavírus

RLH – *RIG-like Helicase* – Helicase tipo RIG

SI – Sistema Imunológico

SIL – Lesão Intraepitelial Cervical

SNP – Single nucleotide polymorphism – Polimorfismo de única Base

TH – Linfócito T *helper* – Linfócito T auxiliar

TLR – *Toll-like Receptors* – Receptores Tipo Toll

TNF – *Tumour Necrosis Factor* (Fator de Necrose Tumoral)

UV – Ultravioleta

SUMÁRIO

1 REVISÃO DE LITERATURA

1.1 PAPILLOMAVIRUS

Papillomavirus (PVs) do latim *papila* - projeção ou saliência em forma de mamilo, e da desinência – *oma*, usada pelos antigos médicos gregos para designar as tumorações ou os entumescimentos. Os papillomavirus causam lesões epiteliais tanto em humanos quanto em animais, sendo encontrados em mais de vinte diferentes espécies de mamíferos, aves e répteis. A infecção por estes vírus são espécie específicos (van Regenmortel et al., 2000; Bernard et al., 2005).

Os PVs foram originalmente agrupados juntos com os polyomavirus em uma única família, a *Papovaviridae.* Isto aconteceu baseado em similaridades como, capsídeos não envelopados e o genoma composto de uma única molécula de DNA fita dupla. Posteriormente reconheceu-se que os dois grupos de vírus possuíam diferentes tamanhos e organização completamente diferente dos genomas e não possuíam similaridade na maioria das sequências de nucleotídeos ou sequências de aminoácidos, e, por isso, foram oficialmente reconhecidos pelo – Comitê Internacional de Taxonomia Viral (*The International Committee on the Taxonomy of Viruses* - ICTV) – como duas famílias separadas, A *Papillomaviridae* e *Polyomaviridae* (IARC, 2007).

1.1.1 Genoma, Proteínas e Ciclo de vida

Papillomavirus são vírus pequenos, não envelopados e icosaédricos que possuem um diâmetro de 52-55nm. As partículas virais consistem de uma única molécula de DNA fita dupla de aproximadamente 8000 pares de base (bp) ligadas a proteínas histonas. Este material está contido em um capsídeo proteico composto de 72 capsômeros pentaméricos. O capsídeo contêm duas proteínas estruturais – late

(L1) (tamanho de 55kDa; 80% das proteínas virais) e L2 (70kDa) – as quais são codificadas pelo vírus. O virion intacto tem densidade de 1.34g/mL em cloreto de césio e um coeficiente de sedimentação de 300 (Kirnbauer et al., 1992; Hagensee et al., 1993).

Os genomas de todos os tipos de HPV contêm aproximadamente oito quadros de leitura aberta (ORFs). Essas ORFs podem ser divididas em três partes funcionais: A região precoce (E – *Early*) que codifica proteínas (E1 – E7) necessárias a replicação; a região tardia (L – *Late*) codifica proteínas estruturais (L1 – L2) que são necessárias para a montagem do vírion; e uma grande região não codificante que é conhecida região longa de controle (L*ong Control Region* - LCR), a qual contém elementos cis, que são responsáveis pela replicação e transcrição do DNA viral. As proteínas virais E são transcritas através do promotor “precoce” enquanto as proteínas L são transcritas, principalmente, através do promotor “tardio” (Fehrmann et al., 2003).

As proteínas E1 e E2 do HPV atuam como fatores que reconhecem a origem de replicação; a proteína E2 é também o principal regulador da transcrição do gene viral. Acredita-se que a proteína E4 está envolvida nos estágios finais do ciclo de vida do vírus e E5 pode funcionar tanto na fase precoce quanto na fase tardia. As proteínas E6 e E7 têm como alvo um número de reguladores negativos do ciclo celular, primariamente p53 e pRb, respectivamente. Durante o ciclo viral, E6 e E7 facilita a manutenção estável dos episomas virais e estimula a diferenciação das células fazendo com que elas entrem novamente na fase S. As proteínas L1 e L2 montam os capsômeros, formando capsídeos icosaédricos em torno do genoma viral durante a geração da progênie de virions (Fehrmann et al., 2003).

1.1.2 Classificação

A classificação em genótipos ou tipos baseia-se na comparação de sequências de nucleotídeos do gene L1 dos diversos HPV. Cada tipo de HPV difere dos outros em pelo menos 10% na sequência de nucleotídeos do gene L1. Quando a diferença na sequência desse gene varia entre 2 a 10%, o isolado é considerado subtipo. Se a divergência for menor que 2%, fala-se em variante do tipo (Bernard et al., 1994).

Por causa da sua importância médica, os HPV têm sido extensivamente estudados, e mais de 100 diferentes tipos já foram identificados. Os genótipos são nomeados pela abreviação HPV seguida de um número que é dado sequencialmente, à medida que diferentes tipos são descobertos (Storey et al., 1988).

Os PVs são perfeitamente adaptados ao seu hospedeiro natural, e possuem tropismo específico por células epiteliais escamosas da pele e mucosas, ao qual utilizam a maquinaria celular em benefício próprio. Dos mais de 100 genótipos de HPV já caracterizados, cerca de 30 infectam a mucosa do trato urogenital e anal, podendo causar lesões tanto benignas, quanto malignas (Bernardet al., 2005).

Os HPVs mucosotróficos podem ser categorizados em baixo ou alto risco, de acordo com o seu potencial oncogênico (Storeyet al., 1988; Muñoz et al., 2003). Os HPVs de baixo risco (LR-HPVs) (ex. HPV 6, 11, 42, 43 e 44) estão presentes na maioria das infecções clinicamente aparentes como tumorações benignas, verrugas ou condilomas acuminados. Já condições malignas, que aparecem na forma subclínica, como NIC e o carcinoma invasivo estão associados aos HPV de alto risco e também à coinfecção por HPV de baixo e alto risco (ex. HPV 16,18, 31, 33, 45, 58)

(Delius et al., 1988). Entre os HPVs de Alto Risco (HR-HPVs) os tipos 16 e 18 são os mais prevalentes, sendo encontrados em mais de 70% das amostras de câncer cervical no mundo, por isso, são considerados os principais causadores desta doença e das lesões precursoras (Smith et al., 2007).

Os HPVs mucosotrópicos pertencentes ao gênero alpha, incluindo os tipos de HPV de alto risco 16 e 18, estão associados com mais de 99% dos casos de câncer cervical (Walboomers et al., 1999). Nesses canceres, o genoma do papillomavirus encontra-se frequentemente integrado ao cromossomo do hospedeiro (Boshart et al., 1984; Schwarz et al., 1985; Yee et al., 1985). Células epiteliais cervicais que tem integrado ao seu genoma o DNA do HPV 16 possuem uma vantagem de crescimento quando comparado a outros tipos de HPV contendo genoma viral na sua forma extracromossomial. Esta vantagem de crescimento está associada com o aumento da expressão de dois genes virais em particular, E6 e E7 (Jeon et al., 1995). A expressão dos genes virais E6 e E7 é necessária para o crescimento contínuo de linhagens celulares derivadas do câncer cervical (Nishimura et al., 2000; Wells et al., 2000). Estes fatos suportam a hipótese de que E6 e E7 causalmente relacionados ao início e manutenção de canceres cervicais humanos. Além disso, a expressão contínua dessas proteínas precoces pode levar a acumulação de mutações no genoma celular que são necessárias a carcinogênese (zur Hausen et al.,1999). Tanto E6 quanto E7 cooperam para indução da transformação de células epiteliais (Münger et al., 1989), no entanto, um fenótipo totalmente maligno somente é observado após um cultivo prolongado de células transformadas (Hurlin et al., 1991; Dürstet al., 1995).

1.1.3 Carcinogênese

Por razões desconhecidas, a infecção pelo HPV tende a causar câncer em áreas referidas como “zonas de transformação”. Nestas áreas, são onde ocorre um processo chamado de metaplasia, em que um epitélio foi ou está sendo gradualmente substituído por outro. A cervice e o ânus são exemplos de tecidos que contém essas zonas de transformação aos quais estão mais propensos ao processo de carcinogênese do HPV. No caso da cervice, a zona de transformação é a área onde o epitélio colunar está sendo, ou foi substituído pelo epitélio escamoso metaplásico (Moscicki et al., 2006).

O ciclo de vida produtivo dos HPV está diretamente relacionado à diferenciação celular epitelial. Tem sido sugerido, que para manutenção da infecção, o HPV tem que infectar as células da camada basal, uma vez que estas são as únicas no epitélio escamoso que são capazes de se dividir (Egawa et al., 2003). Para que as partículas virais consigam chegar às células da camada basal, é necessário que haja fissuras ou microlesões no epitélio estratificado. Estas fissuras normalmente ocorrem como resultado, por exemplo, da atividade sexual (Schneider et al., 1994).

Na maioria das mulheres infectadas pelo HPV cervical, as lesões causadas pelo vírus não progridem ao câncer. Porém, em algumas mulheres, as lesões não regridem com sucesso, podendo persistir e progredir ao carcinoma invasivo (Gross et al., 1997). Isto sugere que em torno do HPV orbitam outros fatores que influenciam direta ou indiretamente na instalação desse mecanismo no epitélio escamoso cervical (Muñoz et al., 2006).

Estas lesões foram divididas, histologicamente, em três graus: NIC I, caracterizada por atipias celulares localizadas no terço inferior do epitélio escamoso; NIC II, em que as atipias ocupam os dois terços inferiores desse epitélio; e NIC III, em que as células atípicas comprometem mais de dois terços ou toda a espessura do epitélio (Richart et al., 1968).

Em 1988 foi criado um novo sistema de classificação citológica cervical com terminologia uniforme, com vistas a facilitar o manejo clínico das NIC, sendo o mesmo revisto em 2001 (Solomon et al., 2002). A partir desses estudos e baseado na nomenclatura citológica do Sistema de Bethesda, Richart transportou para a histologia esses conceitos citológicos, classificando as NIC I em lesões intraepiteliais escamosas de baixo grau associadas à infecção pelo vírus do papiloma humano (HPV) e agrupando as NIC II e III em lesões intraepiteliais escamosas de alto grau (Richart et al., 1990).

1.2 CÂNCER CERVICAL

O câncer cervical (CC) é o tipo mais comum de câncer nos países em desenvolvimento e a principal causa de morte por câncer entre as mulheres. A estimativa mundial de novos casos de câncer cervical por ano é de 525.000 (IARC, 2007). O número de casos novos de câncer do colo do útero esperado para o Brasil no ano de 2016 será de 16.340, com um risco estimado de 15,85 casos a cada 100 mil mulheres. Sem considerar os tumores de pele não melanoma, o câncer do colo do útero é o segundo mais incidente na Região Nordeste (19,49/100 mil) (INCA, 2015). Na América Central e na América do Sul a taxa de incidência é cerca de cinco vezes maior que na Europa Ocidental (Arossi et al., 2003).

Além do câncer cervical, o DNA do HPV é encontrado em aproximadamente 10% de todos os cânceres humanos, bem como em condições pré-cancerosas. Cerca de 26% dos cânceres de cabeça e pescoço estão ligados a infecção por HPV (Gillison et al., 2004). Enquanto que as infecções por HPV são comuns em todos os grupos sociodemográficos, alta prevalência foi encontrada entre mulheres não casadas, com baixo nível de escolaridade, que possuem baixa condição socioeconômica e que pertencem a certos grupos étnicos (Dunne et al., 2007).

1.2.1 Fatores de Risco Associados ao Câncer Cervical

A presença de um tipo oncogênico do HPV na região cervical é considerada uma causa necessária para o desenvolvimento da NIC e do CC, mas não exclusivamente. Caracterizada como uma doença multifatorial, o HPV atua em conjunto a cofatores ambientais e fatores imunológicos e genéticos próprios do hospedeiro para que ocorra a progressão para o câncer cervical invasivo (Ylitalo et al., 2000; Bosch et al., 2002;. Craveiro et al., 2004; Santos et al., 2005).

Fatores ambientais relacionados com CC incluem fatores hormonais (uso de contraceptivos orais), multiparidade, coinfecção com outros vírus (herpes simplex 2 - HSV-2) e bactérias ou com outras infecções sexualmente transmissíveis (*Chlamydia trachomatis* e HIV) e hábitos do hospedeiro como fumo, exposição à radiação ultravioleta (UV) e os fatores dietéticos. Outros cofatores não ambientais incluem os relacionados à resposta imune do hospedeiro e as relacionadas com o próprio vírus, como o genótipo de HPV, coinfecção com outros tipos de HPV, as variantes de HPV, carga viral e integração viral (Castellsagué, 2002).

1.3 A IMUNIDADE HUMANA

A resposta imune tem papel fundamental na defesa contra agentes infecciosos e células transformadas, incluindo o câncer. Para a quase totalidade das doenças infecciosas, o número de indivíduos expostos à infecção é bem superior ao dos que apresentam doença, indicando que a maioria das pessoas tem condições de destruir esses microrganismos e impedir a progressão da infecção. Em contraste, as deficiências imunológicas, sejam da imunidade inata ou da imunidade adaptativa, são fortemente associadas com aumento de susceptibilidade a infecções (Janeway, 2001).

As respostas imunes são mediadas por uma variedade de células e moléculas solúveis que estas secretam como resposta a eventos perigosos como patógenos e células transformadas. As principais células efetoras da imunidade inata são: células dendríticas (CDs), macrófagos, eosinófilos, mastócitos, neutrófilos e células *natural killer* (NK). Os componentes humorais incluem proteínas do complemento, proteínas de fase aguda, citocinas, dentre outras (Turvey, 2010).

Tradicionalmente a imunidade inata tem sido vista como a primeira linha de defesa, discriminando o “próprio” (ex. proteínas do hospedeiro) do “não-próprio” (ex. microrganismos). No entanto, a literatura emergente sugere que a imunidade inata, na verdade, atua como um sistema sofisticado para sentir sinais de “perigo”, tais como microrganismos patogênicos ou sinais de estresse celular enquanto não responde a sinais “não perigosos”, tais como, moléculas normais do hospedeiro, antígenos alimentares ou da microbiota comensal do intestino (Schroder, 2010).

A imunidade adquirida consiste em linfócitos T e B e seus mediadores humorais, incluindo citocinas e anticorpos. Em contraste com o limitado número de

receptores para patógenos do sistema imune inato, o sistema imune adquirido possui um repertório extremamente diversificado de receptores, gerados aleatoriamente através de rearranjos somáticos nos genes (Maruyama et al., 2010).

A resposta da imunidade adquirida à primeira exposição ao patógeno é lenta e demora geralmente cinco dias para expansão clonal dos linfócitos T e B antígeno específicos. Porém, a imunidade adquirida é capaz de gerar memória imunológica, respondendo de forma mais rápida se houver uma segunda exposição ao patógeno (Bonilla, 2010).

1.3.1 Receptores e Integração

O sistema imune inato envolve uma série de receptores de reconhecimento padrão (PRRs) codificados nas células germinativas para detectar padrões microbianos invariáveis. Os PRRs são expressos por células da primeira linha de defesa contra infecções, incluindo macrófagos, monócitos, células dendríticas, neutrófilos e células epiteliais, bem como células do sistema imune adaptativo. Nesta família estão inclusos os receptores ligados a membrana “Receptores tipo Toll” (Toll-like Receptors - TLRs) e lectinas tipo C (CTLs), as quais varrem o meio extracelular e o compartimento endossomal a procura de padrões moleculares associados a patógenos (PAMPs). Os PRRs sensíveis a ácidos nucleicos intracelulares cooperam para prover uma varredura citosólica, incluindo as helicases tipo RIG (RLHs) sensíveis a RNA, RIG-I e MDA5, e os sensores de DNA, DAI e AIM2. O resultado do reconhecimento de uma PAMP pelos PRRs depende da natureza tanto da célula respondente, quanto do organismo invasor. No entanto, sinais de transdução a partir desses receptores convergem para um conjunto comum de módulos sinalizadores, frequentemente incluindo a ativação de fatores de

transcrição NF-κB e AP-1 que direcionam a produção de citocinas/quimiocinas pro-inflamatórias e membros da família do fator de transcrição iRF que media respostas antivirais interferon (IFN) tipo I dependentes. Outro conjunto de PRRs intracelular, distintos dos descritos acima, são os receptores tipo NOD (NLRs) que reconhecem PAMPs, bem como sinais de perigo derivado do hospedeiro (danger-associated molecular patterns, DAMPs) (Schroder, 2010).

Através de manifestações de inflamação e morte ou dano celular, efetores celulares e humorais da imunidade inata são ativados e recrutados para o local. As CDs agem como células apresentadoras de antígeno (APCs), e se tornam ativadas ao encontrarem o agente infeccioso. Elas fagocitam, processam e expressam o antígeno em um receptor da superfície celular chamado de complexo de histocompatibilidade principal de classe dois (MHC II), e depois apresentam o antígeno para os linfócitos (Delves, 2000).

Os linfócitos T utilizam seus receptores antígeno-específicos (TCRs) para reconhcer os peptídeos antigênicos ligados às moléculas do MHC. Os dois principais tipos de linfócitos T são indentificados pelos de marcadores de superfície da célula CD4 ou CD8. Os linfócitos T CD4+ reconhecem atígenos apresentados pelo MHC de classe II que são expressos por APCs; Os linfócitos T CD8+ reconhecem antígenos apresentados pelo MHC de classe I que são expressos por células infectadas que se marcam para ser o alvo da citotoxidade destes linfócitos (Janeway, 2001).

Os linfócitos T CD8+ possuem atividade citotóxica, como citado anteriormente, enquanto os linfócitos T CD4+ possuem função de regulatória da atividade de outras células através da secreção de mediadores solúveis chamados

de citocinas. Os linfócitos T CD4+ podem ser divididos em tipos, de acordo com o perfil de secreção de citocinas (WU, 1994):

- Th1: ENTRE ELAS Secreta a interleucina 2 e o interferon gama que facilita a resposta mediada por células, incluindo a ativação de macrófagos, células NK e linfócitos T CD8+, auxiliando-os na destruição de patógenos intracelulares

- Th2: Secreta as interleucinas 4, 5, 6 e 10, que ajudam na diferenciação dos linfócitos B em plasmócitos e na secreção de anticorpos.

- Th17: Derivam de linfócitos T CD4+ Naïve na presença de TGF-β e IL-6 (Aggarwal 2003) e estão envolvidos na patogênese de várias doenças humanas, incluindo doenças autoimunes, inflamatórias, alergias e cânceres (Ye et al., 2013; Bailey et al., 2014). IL-17

As citocinas têm um papel central na imunidade, pois elas determinam que tipo de resposta a ser dada para fornecer uma ótima proteção ao hospedeiro contra agentes infecciosos específicos (Janeway, 2001).

As citocinas são um diversificado grupo de proteínas ou glicoproteínas solúveis que fazem parte de uma rede de sinalização, mediando interações entre células. Elas podem ser agrupadas em várias subfamílias: Interleucinas (numeradas de IL-1 a IL-26), interferons (IFNs), fatores estimuladores de colônia (CSFs), fatores de necrose tumoral (TNFs), fatores de crescimento (TGF) e quimiocinas. Esta nomenclatura é um pouco arbitrária, pois as citocinas possuem múltiplas funções, podendo assim, possuir ações semelhantes, de modo que existe uma redundância em, pelo menos, alguns de seus papéis (Dunlop, 2000; Hopkins, 2003).

As citocinas são liberadas por provavelmente todas as células humanas em concentrações diminutas, formando importantes proteínas “mensageiras” solúveis. Atuam geralmente em pequenas distâncias, de maneira autócrina (agindo sobre as próprias células) ou parácrina (agindo sobre células próximas), com exceção de uma minoria que pode ter uma ação sistêmica, como a IL-6. Elas interagem com receptores específicos das células ligados a um segundo mensageiro intracelular, ao qual estabelece uma cascata de reações que leva à indução ou à inibição da transcrição de inúmeros genes por vias de sinalização celular (Dunlop, 2000; Hopkins, 2003).

Diferente dos hormônios endócrinos que são produzidos diariamente com a finalidade de assegurar o funcionamento eficiente dos órgãos, a produção de citocinas é transitória e rigorosamente controlada. Elas têm um papel fisiológico na restauração da função normal dos tecidos, quando estes são submetidos a desafios importantes ou mudanças, como em eventos que ocorrem normalmente durante infecções ou traumas (Dunlop, 2000).

Assim, além de ter um papel importante na defesa e reparação dos tecidos, as citocinas também têm a função de controlar as respostas da imunidade inata e adquirida, incluindo: inflamação, defesa contra infecções virais, proliferação e controle das funções diferenciadas dos linfócitos T e B.

O estímulo clássico para a produção de citocinas é através da interação dos PAMPs com os receptores *Toll-like* das células da imunidade inata. Estes receptores desencadearão uma cascata de sinais intracelulares, e resulta na transcrição de citocinas pró-inflamatórias, como IL-1, IL-6 e TNF-α que são capazes de induzir a inflamação (Shalaby et al., 1989).

Embora a inflamação seja um processo autolimitado que atua na defesa do organismo contra infecções ou danos, a resolução inadequada das respostas inflamatórias geralmente leva a doenças crônicas, incluindo o câncer (Schottenfeld, 2006). Extensivos estudos sobre polimorfismos genéticos têm sido descritos, e em muitos casos, estes polimorfismos aumentam os níveis das citocinas que induzem a inflamação, estando ligado a uma variedade de doenças (Bidwell et al., 2001).

1.3.2 Inflamassomas e Citocinas

1.3.2.1 *Inflamassomas*

Inflamassomas são plataformas multiproteicas cujas moléculas possuem uma massa molecular de pelo menos 700kDa (Martinon, 2002), elas controlam a ativação da cisteinil-aspartato protease caspase-1 e a clivagem da pro-il-1b, permitindo a liberação da citocina ativa madura de 17-kDa (Meylan, 2006; Franchi et al., 2009). Caspases são responsáveis por aspectos cruciais da inflamação e morte celular e podem ser amplamente divididas em duas classes baseadas na sua especificidade ao substrato: as pro-apoptoticas e as pró-inflamatórias. Caspase-1 faz parte do grupo das pro-inflamatórias juntamente com a caspase-4, caspase-5, caspase-11 e caspase-12 (Siegel, 2006). A necessidade de dois diferentes estímulos para regular a produção de IL-1b garantem que a IL-1b (ou a citocina IL-18) não seja incorretamente liberada, já que isso pode gerar consequências deletérias para o hospedeiro. Na verdade, o excesso de produção da IL-1b está associado com síndromes hereditárias de febre periódica, bem como doenças autoimunes e inflamatórias, tais como a gota ou artrite reumatoide (McDermott, 2007; Masters et al., 2009).

Muitos complexos inflamassomas usados para ativar caspase-1 têm sido descritos até o momento, tais como receptores tipo NOD contendo domínio pirina –

NLRP1 e NLRP3, IPAF e AIM2 (Martinon, 2009; Schroder, 2010, entre as quais a NLRP3 é mais bem estudada.

NLRP3 é uma proteína de 1016 aminoácidos transcrita a partir do gene cias1, o qual está localizado no cromossomo humano 1q44 e consiste de 9 exons. A arquitetura da NLRP3 assemelha-se a do subconjunto de genes resistentes a doenças de plantas e envolvido na resposta hipersensível contra patógenos virulentos (Lamkanfi, 2009). NLRP3 compartilha a presença de um domínio NOD central (também chamado de domínio NBD ou NACHT) com outros membros da família NLR (Kanneganti, 2007).

O Nlrp3-inflamassoma - NALP3 - é capaz de reconhecer padrões citidina-fosfato-guanosina no DNA presente em bactérias e vírus e são capazes de detectar ssRNA, dsRNA e alguns vírus (influenza vírus, Sendai vírus, adenovírus e HIV) (Martinon, 2009; Thomas et al., 2009; Allen et al., 2009; Hornung et al., 2009; Pontillo et al., 2010).

Polimorfismos naturais ou variações genéticas entre indivíduos em genes relacionados à imunidade têm se mostrado importantes na suscetibilidade a diversas doenças podem ser de interesse na identificação de fatores de suscetibilidade à persistência da infecção por HPV e na identificação de fatores de suscetibilidade à progressão para o carcinoma. (Wang, 2003).

Polimorfismos de base única (SNP) no gene da NLRP1, localizado no cromossomo humano 17p13.2, foram relacionados com diversas desordens imunes, tais como doenças autoimunes múltiplas relacionadas ao vitiligo (Jin, 2007) e com a doença autoimune de Addison e diabetes tipo 1 (Magitta, 2009). Já os SNPs no gene da NLRP3 tem sido associados com desregulações inflamatórias (Verma,

2008), com a estabilidade do mRNA (Hitomi, 2009) e mais recentemente com a suscetibilidade a infecção por HIV (Pontillo, 2010).

Outros componentes do complexo proteico inflamassoma e moléculas associadas ao seu correto funcionamento também podem sofrer interferência de SNPs, como é o caso do domínio de recrutamento associado à caspase – CARD-8 e do Fator Nuclear κB (NF-κB), localizados nos cromossomos 19q13.33 e 13q14, respectivamente. Estudos anteriores demonstraram que a expressão constante do CARD-8 em monócitos THP-1 causou um decréscimo na secreção da IL-1, provavelmente devido à interação física com a caspase-1 (Razmara et al., 2002). Outras funções relacionadas ao CARD-8 incluem a regulação da apoptose (Pathan et al., 2001) e propriedades inibitórias do NF-κB (Razmara et al., 2002; Bouchier-Hayes et al., 2001). Polimorfismos no NF-κB tem sido relacionadas com uma atividade promotora atenuada, o que pode levar a problemas na produção e nos níveis de citocinas pró-inflamatórias (Karban et al. 2004). Além disso, a Proteina Editora de Ubiquitina A20, também conhecida como Proteína 3 induzida pelo Fator de Necrose Tumoral ou TNFAIP3 Tem sido descrita como um fator chave na terminação da sinalização do NF-κB (Vereecke, 2009).

1.3.2.2 *TNF-α*

O Fator de Necrose Tumoral alfa (TNF-α) é uma potente citocina Th1 pró inflamatória liberada principalmente por monócitos e macrófagos estimulados. O gene se encontra dentro do complexo de histocompatibilidade maior no cromossomo 6p21.3 (Kirkpatrick et al., 2004; Duarte et al., 2005), e é composto de quatro éxons dispostos ao longo de cerca de 3 kb do DNA (Laing et al., 2001).

O TNF-α foi originalmente identificado e isolado por duas atividades características: a capacidade de induzir necrose hemorrágica de certos tumores e da capacidade de induzir caquexia durante os estados de infecção crônica (Maqsood et al., 2009). Em infecções severas, o TNF-α é produzido em larga escala e causa anormalidades sistêmicas clínica e patológica (Abbas, 2003).

A principal função fisiológica do TNF-α é estimular o recrutamento de neutrófilos e monócitos para os locais da infecção e ativar essas células para erradicar microrganismos (Abbas, 2003). Possui amplos efeitos biológicos, incluindo proteção contra a infecção, vigilância contra tumores, estimula respostas inflamatórias (Laing et al., 2001), além de desempenhar um papel essencial no sistema imunológico (Barth et al., 1996). O TNF-α estimula a síntese de outras citocinas pró inflamatórias e a adesão de moléculas. Além disso, tem mostrado mediar a carcinogênese através da indução, proliferação, invasão e metástase de células tumorais (Castelletti, 2006). Em situações agudas, a produção local de TNF-α é claramente benéfica. Ele aumenta a expressão de moléculas de adesão no endotélio vascular a fim de permitir que células imunológicas, particularmente os neutrófilos e macrófagos, se desloquem para os sítios onde ocorreram dano tecidual e infecção (Maqsood, 2009).

Polimorfismos genéticos de citocinas, especialmente em regiões reguladoras, podem estar relacionados com a quantidade de citocinas produzida (Nishimura et al., 2001). O nível de produção desta citocina varia com o genótipo do indivíduo que secreta, podendo produzir níveis baixo, médio ou alto de TNF-α.

Existem vários polimorfismos localizados em diferentes sítios do promotor do gene responsável pela síntese da TNF-α Foram identificados na região -1031 (T→C), -863 (C→A), -857 (C→A), -851 (C→T), -419 (G→C), -376 (G→A), - 308 (G→A), -238 (G→A), -162 (G→A) e – 49 (G→A). Polimorfismos de nucleotídeo único nas regiões -308 e -238 do promotor do gene *TNF* têm sido comumente estudados (Kroeger, 1997; Wilson et al., 1997; Elahi et al., 2009).

O polimorfismo na posição -308 afeta a expressão do gene e têm sido implicados na regulação da transcrição do *TNFα*. Podem ser encontradas numa determinada população duas formas alélicas, onde uma permanece com o alelo G, comum, da guanina ou quando ocorre substituição desse alelo pelo A (adenina) (Bouma et al., 1996; Fernandes et al., 2008).

Segundo Cabrera *et al.* (1995) as células que contém o alelo A aumentam a transcrição de seis a nove vezes mais que aquelas que contém o alelo G (Kroeger, 1997; Wilson et al.; 1997; Hildesheim et al., 1998). Outros trabalhos relataram que *in vitro*, o alelo G está relacionado com a reduzida produção de TNF-α, enquanto os indivíduos com alelo A produzem mais TNF-α (Kroeger, 1997; Turner et al., 1995; Braun et al., 1999; Chen et al., 1996).

1.3.2.3 *TGF-β*

O fator de crescimento transformador β1 (TGF-β1), localizado no cromossomo 19 (19q13), é uma molécula fundamental de homeostase entre o crescimento celular e a apoptose. O balanço entre proliferação celular, sobrevivência e morte celular é central para muitos processos fisiológicos e sua desregulação pode induzir doenças. Enquanto o excesso de apoptose é observado em doenças crônicas degenerativas e imunodeficiência, apoptose insuficiente pode participar em processos cancerígenos e de autoimunidade. Ademais, apoptose é um processo crítico na seleção de células T no sistema imune (Gupta, 2000).

De acordo com a ideia de que a proteína TGF-β pode atuar como um promotor tumoral, o mRNA *TGF-β1* aumentado, a expressão de proteínas em células tumorais e/ou seus níveis plasmáticos, têm sido correlacionados com progressão tumoral aumentada nos cânceres coloretal, gástrico, pulmonar e de próstata (Dalal, 1993; Shim et al., 1999; Saito et al., 1999; Hasegawa et al., 2001). Níveis de TGF-β no soro são significativamente maiores em pacientes com câncer de pulmão ou câncer coloretal que tiveram metástase para os linfonodos (Shim et al., 1999; Hasegawa et al., 2001). Além disso, imunomarcação de TGF-β mostra ser mais forte no local de invasão de linfonodos (metástase) quando comparada com o sítio primário do tumor em câncer coloretal e de mama (Dalal, 1993; Picon et al., 1998). Tumores primários que sofreram metástase tem uma marcação mais forte do que aqueles que não sofreram, e a metástase também exibe forte marcação de TGF-β. Esta é uma forte evidência que sugere que o TGF-β é um fator importante que promove a metástase em fases tumorais tardias. Esse fato está correlacionado com a habilidade do TGF-β em induzir a transição epitelial – mesenquimal, invasão e migração tanto de células não transformadas como de células tumorais in vitro

(Bhowmick et al., 2001; Janda et al., 2002). Altos níveis de TGF-β em tumores também têm sido correlacionados com angiogênese. A expressão de TGF-β é associada com alta densidade vascular em câncer de próstata (Wikstrom et al., 1998) e altos níveis plasmáticos de TGF-β em pacientes com carcinoma hepatocelular está correlacionado com a vascularização tumoral (Ito et al., 1995).

O gene da TGF-β1 possui muitos polimorfismos, incluindo 988 C/A, 800 G/A e 509 C/T na região promotora, inserção (C) na região não traduzida e C263T, T869C, G915C na região codificante (Cambien et al., 1996). Entre eles, três polimorfismos: 509 C/T, T869C e G915C têm sido associados com o nível sérico de TGF-β1 (Awad et al., 1998; Grainger et al., 1999). O SNP T869C está localizado no códon 10 do exon 1 e resulta numa mudança da leucina para prolina, enquanto o SNP G915C está localizado no códon 25 e resulta numa mudança de arginina para prolina. Estudos têm mostrado que o alelo variante C do SNP T869C e o alelo selvagem G do SNP G915C estão associados com um aumento na produção do TGF-β1 (Awad et al., 1998; Dunning et al., 2003; Kaklamani et al., 2005). Esses polimorfismos estão ligados a uma grande variedade de doenças e cânceres humanos, tais como o câncer de mama, câncer cervical, câncer de pulmão e câncer gástrico (Stanczuk et al., 2002; Shu et al., 2004; Kang et al., 2006; Jin et al., 2007).

1.3.2.4 *IL-1*

IL-1, localizada no cromossomo humano 2q14, é uma citocina inflamatória chave que media efeitos profundos em, virtualmente, todos os órgãos do corpo (Fitzgerald, 2000). A família da interleucina 1 (IL-1) é constituída por três proteínas relacionadas, duas agonistas, IL-1α e lL-1β e uma antagonista, IL-1Ra. Os genes para estas citocinas estão agrupados dentro de um segmento de 430kb presente no braço longo do cromossomo 2. As agonistas compartilham uma estrutura terciária muito parecida e partilham dos mesmos fatores regulatórios, afinidades e atividades biológicas.

A IL-1Ra é um inibidor competitivo de IL-1α e lL-1β (Dinarello, 1997; Oppenheim, 2004; Duque et al., 2007). Essas proteínas, produzidas por todos os tipos de células nucleadas principalmente por monócitos-macrófagos, linfócitos B, NK, células dendríticas e endoteliais, são conhecidas como os reguladores-chave da inflamação e da resposta imunológica e atuam como citocinas multifuncionais, podendo afetar todos os tipos de células (Shon, 2003).

Tanto a IL-1α quanto a lL-1β são citocinas que ampliam a cascata inflamatória por ativar células T, regulando a manifestação de outras moléculas de adesão e induzindo a produção de outras citocinas proinflamatórias (como a IL-2) e proteínas associadas (Dinarello, 1996). Promovem, também, o crescimento e a diferenciação das células B, ativam neutrófilos e macrófagos, estimulam a hematopoiese e produzem uma variedade de efeitos sobre tipos celulares não hematopoiéticos. Contudo, sua principal função reside em sua capacidade de intensificar a ativação dos linfócitos T_h por células apresentadoras de antígeno (APC) (Dinarello, 1996; Dinarello, 1997; Oppenheim, 2004; Duque et al., 2007).

A inflamação e, em particular, a inflamação crônica causada pela resposta do hospedeiro pode resultar em estresse oxidativo, contribuindo para a promoção e progressão de tumores (Coussens, 2002). Além disso, citocinas pro inflamatórias, como a IL-1α e β, podem, nas fases iniciais da carcinogênese, induzir fatores de crescimento para as células pré-malignas e causar a produção de intermediários reativos de oxigênio, que são mutagênicos para as células (Dinarello, 1996).

Devido à importância desta citocina na resposta imunológica, estudos epidemiológicos moleculares vêm investigando a associação entre os polimorfismos desta citocina, em especial a IL-1β, e o risco de câncer em diferentes locais, como pulmão, fígado, linfomas, entre outros (Matsuo et al., 2001; Wang et al., 2003; Zienolddiny et al., 2004; Campa et al., 2005).

1.3.2.5 *IL-6*

Interleucina-6 (IL-6) ou Interferon Beta-2 (IFNB2), uma glicoproteína contendo 212 aminoácidos, é uma citocina pleiotrópica imunorregulatória que ativa a maquinaria de sinalização da superfície celular composta de IL-6, IL6RA e o sinalizador compartilhado gp130 (Boulanger et al., 2003). É produzida por uma variedade enorme de células, e desempenha um papel central na defesa do organismo. Esta citocina está envolvida em diferentes processos fisiológicos e patofisiológicos, tais como, metabolismo ósseo, hematopoese, diferenciação e/ou ativação de macrófagos e células T, crescimento e diferenciação terminal das células B, síntese de CP (proteína C-reativa) e carcinogênese (Asschert et al., 1999; Diehl, 2002;).

A expressão da IL-6 é induzida como uma resposta a estímulos inflamatórios de IL-1 e TNF-α (Snick, 1990). Por sua vez, a IL-6 induz quimiocinas e aumenta o número de moléculas de adesão nas células endoteliais, colaborando na geração de respostas inflamatórias (Romano et al., 1997). Além disso, a IL-6 também modula a expressão de genes envolvidos na progressão do ciclo celular e inibição da apoptose (Lin, 2007).

A presença da IL-6 nos tecidos não é uma ocorrência anormal, mas sua produção sem controle leva a uma inflamação crônica subsequente, sendo elevados níveis desta citocina associado com o desenvolvimento de diversas doenças malignas incluindo diferentes tipos de câncer (Culig et al., 2005; Hong, 2007). A IL-6 é um fator de crescimento para o mieloma múltiplo, carcinoma de células renais, linfomas não-Hodgkin, câncer de bexiga e câncer colorretal (Aggarwal et al., 2006),

também atuando conjuntamente com outras citocinas na produção de outros sinais promotores de tumor (Farrow, 2002; Philip, 2004).

Resultados experimentais sugerem que este papel se dá pela regulação aumentada da expressão de receptores de adesão em células endoteliais, tais como Molécula de adesão intercelular-1 e Molécula de adesão de leucócitos-1, e pela estimulação da produção de fatores de crescimento, tais como, fator de crescimento de hepatócitos e fator de crescimento endotelial vascular (Hutchins, 1994; Cohen, 1996; de Jong et al., 2001). Muitos estudos clínicos encontraram que, em diferentes tipos tumorais, um alto nível sérico da IL-6 esteve associado com o avanço no estado da doença (Mouawad et al., 1996; Oka et al., 1996; Zhang, 1999; Shariat et al., 2001) e com um mau prognóstico (Berek et al., 1991; Wu et al., 1996; Zhang, 1999).

O gene da IL-6, localizado em humanos no braço curto do cromossomo 7 (7p21), apresenta um SNP na região promotora (-174 G/C) o qual parece estar associado com variações na expressão da IL-6 e nos níveis séricos (Fishman et al., 1998). Aumento nos níveis de citocina ocorreram no genótipo GG tanto em estudos “in vitro” quanto em estudos “in vivo” (Fishman et al., 1998; Bonafé et al., 2001; Giacconi, 2004), embora alguns autores descrevam valores aumentados para o genótipo CC (Jerrard-Dunne et al., 2003; Haddy et al., 2005). No entanto, é importante levar em consideração que a IL-6 tem uma meia-vida curta e os ensaios envolvendo a mesma são complexos (Neal, 2008).

Este SNP também está associado com o prognóstico de câncer gástrico (de Vita et al., 2001), carcinoma de células renais (Tsukamoto et al., 1992) e câncer de

próstata (Nakashima et al., 2000). No câncer cervical, a IL-6 parece estar envolvida na progressão do tumor e metástase (Kinoshita, 1999; de Vita et al., 2001).

1.3.2.6 IL-8

As quimiocinas (linfocinas secretadas pelos linfócitos T ativados) possuem poderosa atividade quimioatraente para monócitos e neutrófilos. Dentre elas, a interleucina 8 (IL-8) foi a primeira quimiocina descoberta. Esta possui atividade proinflamatória, é produzida no decorrer de reações imunológicas ou inflamatórias agudas, bem como em processos inflamatórios crônicos, uma vez que atrai e ativa neutrófilos em regiões inflamatórias (Oppenheim, 2004; Campa et al., 2005).

A IL-8 é sintetizada como um precursor de 99 aminoácidos, segregado após clivagem de uma sequência de sinal de 20 resíduos, e processada pela clivagem repetida de um N-terminal rendendo diversas variantes biologicamente ativas (Baggiolini, 1992). A forma principal é composta por 72 resíduos de aminoácidos com uma massa molecular de 8,383 daltons e quatro cisteínas que formam duas pontes dissulfeto. A IL-8 possui efeitos biológicos pleotrópicos bem estabelecidos, que incluem a ativação de neutrófilos, quimiotaxia, alteração da forma celular, exocitose de vesículas secretoras e grânulos azurófilos, expressão de moléculas de adesão da superfície, produção de superóxido e de peróxido de hidrogênio, metabolitos reativos de oxigênio e a libertação de matriz de células reabsorvendo enzimas gelatinase e elastase (Baggiolini, 1992). Esta interleucina também é segregada em grandes quantidades por células humanas osteoblásticas normais e por células estromais da medula óssea (osteoprogenitor HBMS) em resposta a IL-1β e TNF- α (Chaudhary, 1996).

Apesar da grande importância desta interleucina na resposta imunológica, poucos estudos têm procurado identificar a participação de polimorfismos desta

citocina frente a algumas patologias, como nefropatia e diabetes tipo 2 (Ahluwalia et al., 2009), câncer de colo (Lurje et al., 2008) e câncer colorretal (Landi et al., 2003).

1.3.2.7 *IL-18*

A Interleucina 18 (IL-18) exerce atividade tanto na resposta inata como na imunidade adaptativa (Okamura et al., 1995; Tsutsui et al., 1996; Okamura et al., 1998; Dinarello, 1999). Pertence à superfamília IL-1 de citocinas próinflamatórias e o gene *IL18* está situado na região 11q22.2-22.3 (Okamura et al., 1998). Anteriormente denominada como fator de indução do interferon, devido a identificação desta propriedade (Parnet et al., 1996), sabe-se que tem ação pleiotropica induzindo a expressão de quimiocinas e citocinas, além de moléculas de adesão como a IL-8 e TNF-α (Kohka et al., 1998; Park et al., 2001; Puren et al., 1998; Wood et al., 2005).

Em cultura de adipócitos humanos pode-se verificar que a IL-18 é secretada pelas células de gordura e a TNF-α estimula a expressão de IL-18 e seus receptores semelhantes aos seus efeitos sobre a inflamação em outros genes relacionados (Wang, 2005)**.**

A IL-18 desempenha um papel primordial na resposta imune, sendo responsável pela diferenciação e ativação das células T helper (Th) em subgrupos, de acordo com o perfil de citocinas (Gracie, 2003). Atua como um indutor para a diferenciação e proliferação de células Th1 na presença de IL-12, estimulando a produção de IFN e TNF nos linfócitos T e natural killer (NK) (Esfandiari et al., 2001; Nakanishi et al., 2001; Gracie, 2003). A IL-18 e a IL-12 atuam em sinergismo na ativação de células NK, células B e T (Torre et al., 2002; Perkmann et al., 2005). Em contrapartida, a IL-18 pode ainda estimular a resposta imune do tipo Th2, na ausência da IL-12 (Tominaga et al., 2000; Nakanishi et al., 2001).

Vários polimorfismos no promotor do gene *IL18* têm sido associados com diferentes doenças inflamatórias e auto-imunes (Tamura et al., 2002; Sivalingam et al., 2003; Tiret et al., 2005; Imboden et al., 2006; Mojtahedi et al., 2006; Sugiura et al., 2006). Cinco posições diferentes de polimorfismos de único nucleotídeo na região promotora foram identificados: -656 G / T, -607 C / A, -137 G / C, 113 G / T, e 127 C / T (Giedraitis et al., 2001). No entanto, somente em SNPs nas posições -137 e -607 foram confirmados como tendo um impacto sobre a atividade do gene *IL18* em estudos anteriores (Kalina et al., 2000).

2 OBJETIVOS

2.1 OBJETIVO GERAL

– Relacionar polimorfismos em genes ligados à imunidade com a infecção pelo Papillomavirus Humano e com a progressão da Neoplasia Intraepitelial Cervical em mulheres atendidas em hospitais de referência da região metropolitana de Recife-PE.

2.2 OBJETIVOS ESPECÍFICOS

– Investigar a relação entre SNPs dos genes relacionados ao inflamassoma, IL-1B, IL-18, TNFAIP3, CARD-8, NLRP1, NLRP3, NLRP6,na susceptibilidade a infecção pelo HPV e progressão de lesões pré-cancerosas;

– Investigar a relação entre SNSPs nas citocinas IL-6, IL-8, TNF-α, TGF-β na susceptibilidade a infecção pelo HPV e progressão de lesões pré-cancerosas;

– Investigar a possível associação entre o polimorfismo +3953 C/T no gene da IL-1 com o risco de infecção por HPV e desenvolvimento de neoplasia intraepitelial

cervical, além de analisar esta relação com alguns fatores de risco, como o consumo de álcool, fumo e uso de contraceptivos orais.

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APÊNDICE A – ARTIGO SUBMETIDO

Submetido: Periódico *Human Immunology*

A dual role of *IL1β* gene +3953 (C/T) polymorphism in HPV infected women with and without cervical lesions.

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ABSTRACT

Human papillomavirus (HPV) have been associated with development of cervical intraepithelial neoplasia (CIN) and cervical cancer (CC) worldwide. Behavioral characteristics and immunologic response play a role in protecting individual against viral infections. This study aimed to investigate the association among *IL-1β* +3953 (T/C) polymorphism and the risk to HPV infection and development of precancerous lesion as well as co-factors role. We analyzed 523 DNA samples from women who attended at the Public Health Units from Pernambuco-Brazil. 135 samples were infected by HPV, 176 infected and presented CIN and 212 not infected neither had CIN. *IL-1β* polymorphism was evaluated through PCR-RFLP method. A protective effect for T allele patients [OR 0.58; p=<0.0001] was observed. Nevertheless, according to the CIN presence an increased risk of 3.6 fold for T allele patients was found [p=0.0001]. Analyzing combined effect of IL-1*β* and behavioral characteristics an increased risk for alcohol consumption [OR=5.87], tobacco smoking [OR=6.96], and OC use [OR=29.9] were observed. Thus, it suggests that T allele patients could have protection against HPV infection, nevertheless once infection is installed these patients could have a risk of developing precancerous lesions increased. Therefore, alcohol, smoking, and OC use may have an important role in the carcinogenic process.

Keywords: Cervical Intraepithelial Lesion; Invasive Cervical Cancer; PCR-RFLP; Cytokines

1. INTRODUCTION

Cervical cancer is the second most common malignant tumor in the world affecting ~ 530 000 new women per year, and approximately half of them will die as a consequence of the disease. Developing countries have 80% of the new cases, making this cancer the most incident in women. Cancers caused by human papillomavirus (HPV) infection represents nearly 5% of all types of cancers [1, 2].

Mucosal HPV infections are the most common sexually transmitted infections in the world [3, 4] and associate with a variety of diseases that range from benign genital warts to cervical and anogenital carcinoma in males and females [4]. According to phylogenetic data and based on their oncogenic potential, HPV infecting the genital tract can be classified into two groups: low-risk (e.g., types 6, 11, 42, 43 and 44) and high-risk HPV such as 16, 18, 31, 33, 35, 39, 45, 52 and 56 being the most prevalent types 16, 18, 31 and 33 [5].

Epidemiological studies have shown that only a small fraction of women infected with oncogenic HPV types will eventually progress to high-grade intraepithelial lesions (HSIL) and cervical cancer (CC). Since infection by oncogenic HPVs is a necessary but not a sufficient cause of CC (6), association between environmental cofactors (such as use of Oral Contraceptives (OC), tobacco smoking, and alcohol consumption), immunological or genetic and HPV infection is directly related to the development of cervical intraepithelial neoplasia (CIN's) and its progression to invasive cervical cancer (ICC) [7-9]. Other non-environmental cofactors being also considered include those related to the host's immune response and those related to the virus itself, such as HPV genotype, co-infection with other HPV types, HPV variants, viral load, and viral integration.

Factors such as cytokines involved in initiation and regulation of the inflammatory response, play an important role in the defense against viral infection. Inter-individual differences in gene expression can be attributed to variants located in the regulatory or other promoter regions of the cytokines genes which may affect the intensity and/or quality of the immune response [10]. Interleukine-1β (IL-1β), located on chromosome 2q14, a proinflammatory cytokine mainly produced by tissue macrophages and blood monocytes, has been implicated in mediating both acute and chronic inflammation [11]. The *IL-1β* gene has three biallelic polymorphisms at positions –511, –31, and +3953 from the transcriptional start site, showing complete

linkage disequilibrium [12, 13]. The single nucleotide polymorphisms (SNP) at position +3953 of the *IL-1β* gene [14], has been associated with several pathologies, such as lumbar disc herniation [15], preeclampsia [16], rheumatoid arthritis [17], periodontitis [18] and coronary artery disease [19] and cervical cancer [20].

All this considered, we decided to investigate the possible association between +3953 C/T polymorphism with risk to HPV infection and the development of cervical intraepithelial lesions in women infected by HPV from Pernambuco, Northeast Brazil. Moreover, we analyzed the possible association with behavior characteristics, as alcohol consumption, tobacco smoking and OC use.

2. MATERIALS AND METHODS

2.1 Samples Collection and Locations

We carried out a population-based cross-sectional study to compare the frequency of HPV infection and *IL-1β* +3953 C/T polymorphism in DNA samples from patients who underwent Pap smear screening attending in Women's Laboratory, an integral unit of the Central Laboratory of Public Health of the State of Pernambuco (LACEN) at the Clinic of Pathology of the Lower Genital Tract (PTGI) and the Center for Women and Nursing Care of Gynecologic Oncology of the Instituto de Medicina Integral Professor Fernando Figueira (IMIP) during the period from January 2008 to April 2012. Informed consent was obtained from all participants (CEP/CCS/UFPE Nº 355/08). After informed consent was obtained, each subject was personally interviewed to obtain information on behavior characteristics as tobacco smoking, alcohol consumption and OC use.

2.2 Study Population

A total of five hundred and twenty-three women from Pernambuco (Northeast Brazil), with ages between 16–75 years, mean age 32.9± 10.8 years were eligible for this study. The study group was stratified in women presenting HPV infection without any lesion (135 women, mean age 40.69 ± 12.76 years); women presenting either low-grade squamous intraepithelial lesion (LSIL) or high-grade squamous intraepithelial lesion (HSIL) (176 women, mean age 33.92 ± 10.83 years). Two hundred and twelve unrelated women volunteers from Pernambuco, aged between 14–70 years (mean age 37.7±10 years), with no history of lesions or neoplastic disease as evaluated by the physician were enrolled as controls and written informed consent was obtained.

Patients were included in the study if there was no discrepancy between their cytological abnormality and the histological diagnosis made at the first visit. All patients were initially assessed by colposcopy and subsequently cervical smears were collected. Histological diagnosis was made according to Solomon et al. (2002) [21] and “Associação Brasileira de Ginetoscopia” (2002) [22]. Moreover, patients

were also stratified according to smoking, alcohol consumption, and use of oral contraceptives.

2.3 Genotyping

IL-1β gene was assessed using the oligonucleotides 5′-CTCAGGTGTCCTCCAAGAAATCAAA-3′ and 5′-GCTTTTTTGCTGTGAGTCCCG-3′ flanking the region +3953 of exon 5 of IL1β and then the genotypes were identified by RFLP analysis using *TaqI* restriction enzyme [16 Reyna 2009].

From the samples studied, 10% of randomly chosen samples were confirmed for polymorphism by direct sequencing using the ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

2.4 Statistical analyzes

Univariate statistical analysis was performed using the BioEstat 5.0 software. The study was cross-sectional, with independent samples consisting of nominal data (genotypes). The influence of the polymorphism on the risk for development of (pre) neoplastic cervical disease was estimated by odds ratio (OR) and a 95% confidence interval (CI). Allele frequencies were estimated by direct counting. Comparison between genotypic frequencies of patients and control groups was performed by $\chi 2$ test and Fisher's exact test was used to compare the allele frequencies in contingency tables.

For identification of relevant risk factors, a logistic regression analysis was carried out (comparing HPV-positive women with history of lesions with HPV-positive women with no history of lesions or cervical cancer). The OR and their respective 95% CI were determined. All p values under or equal to 0.05 were considered statistically significant.

3. RESULTS

Five hundred and twenty-three eligible women were enrolled in this study. Of these 59.46% (311) were HPV-infected (HPV+). Table 1 shows the distribution of genotypes and allele frequencies for *IL-1β* +3953 (C/T) polymorphism in HPV positive and healthy control groups. In the control group, genotype frequencies and allele frequencies did not deviate significantly from those expected according to the Hardy-Weinberg equilibrium (p = 0,722). However, the same was not observed in HPV positive group (p < 0.0001). A significant difference in the distribution of *IL-1β* +3953 (C/T) polymorphism between cases and controls was observed, using CC genotype and C allele as reference, as for TT genotype [OR=0.14; 95%CI (0.06-0.35); p<0.0001] as for T allele [OR=0.58; 95%CI (0.44-0.76); p<0.0001] being this variant associated with protection to HPV infection.

The group HPV positive was stratified according to the presence of cervical lesion (Table II). An increased risk to develop cervical lesions (3.76 fold) was observed in the presence of CT genotype (OR: 3.76; CI: 2.30 – 6.14; p=<0.0001). Using dominant model, genotypes that carry at least one T had 3.6 fold more risk to develop cervical lesions (OR = 3.60 CI 2.23 – 5.82, p=<0.0001).

Furthermore, individuals of HPV+ group were stratified according to the degree of cervical intra-epithelial neoplasia: 20.49% (25/122) of patients had LSIL, 70.5% (86/122) had HSIL and 9.02% (11/122) had ICC. No significant differences were observed in relation to progression of cervical lesions for both allele and genotype frequencies (p> 0.05) (Table III).

Three risk factors, namely smoking, alcohol consumption, and use of oral contraceptive (OC) were evaluated for association with risk of developing cervical lesions. When considering subjects HPV+ without lesion, 27.27% (36/132) reported smoking, 46.21% (61/132) reported alcohol consumption, and 14.4% (19/132) reported use of OC (Table IV). While regard individuals HPV+ with lesion, 30.2% (69/176) reported smoking, 57.92% (102/176) reported alcohol consumption, and 59.09% (104/176) reported use of OC.

Significant differences were observed between HPV+ without lesion and HPV+ with lesion groups, for smoking and use of oral contraceptive using univariate analysis (OR= 1.7196; CI: 1.0554 - 2.8018; p= 0.039 and OR= 8.59; CI: 4.8515 -

15.2116, p=<0.0001, respectively), these data suggest that alcohol consumption and use of OC associate with an increased risk for development of cervical lesions.

When comparing the presence of both CC or CT+TT genotypes with each risk factor analyzed in the groups with and without cervical lesions, it was observed a significant rise in risk to develop cervical lesions in patients carrying the CC genotype that reported take oral contraceptive (OR = 1.09; CI: 3.8339 - 17.0360, p= <0.0001) and in all three factors in patients with CT+TT genotypes, namely smoking (OR = 6.963 CI 3.1279 - 15.5002, p= <0.0001), alcohol consumption (OR= 5.8712 CI 2.9046 - 11.8679, p= <0.0001), and oral contraceptive (OR= 29.9063 CI 10.5975 - 68.3130, p= <0.0001).

4. DISCUSSION

The host immune system plays an important role in the development of cervical HPV-related cancers, and cytokines have been frequently correlated with the risk of cervical cancer. Although many women are infected with high-risk types of HPV, only a subset of infected women develops cervical cancer, suggesting that other cofactors must be present for the development of malignancy [23].

The intrinsic ability to produce variable quantities of IL1β during the various stages of clinical cervical lesions can be of great importance in the immune pathogenesis [24, 25]. Several studies showed that genetic polymorphisms in regions +3953 C/T, -511 T/C and -31 T/C of the *IL1-β* gene are able to influence the resistance to infections and susceptibility to various diseases [15, 20, 26, 27], some research facing to relationship between *IL-1β* +3953 polymorphism and production of *IL-1β* by leukocytes [28-30].

So, in the context of risk factors involved in the susceptibility to HPV infection, it was used the more common C allele of the region +3953 *IL-1β* gene as reference. In this study, the less common T allele was more frequent in healthy patients than HPV infected patients (35% vs. 24%, respectively) (OR=0.58; CI: 0.44 – 0.76, p=<0.0001), suggesting that this allele confers protection to HPV infection. A high production of IL-1β was already linked to protection against certain viruses, like Influenza virus [31], Herpes simplex virus-1 [32], and Human Rhinovirus [33]. Moreover, Pontillo et al. (2012) [34] found an increased risk to HIV infection associated to 1β low producers.

To our knowledge this is the first study that sought to associate *IL-1β* +3953 C/T polymorphism with HPV infection, even if some studies relating *IL-1β* with immune response against HPV-associated cervical cancer have been published so far [24, 35, 36].

Our results may be explained due the fact of T allele confers to cells a higher production of IL-1β cytokine, which may act protecting patients from HPV infection, being expressed through inflammasome activation pathways.

Nevertheless, our study showed 3.6 fold increased risk to development of cervical lesion for T allele carriers (p=<0.0001) when we compare HPV infected patients with and without cervical lesions (Table II). Significant associations of this polymorphism were observed in different populations, as northern Indians for cervical

cancer [20] or Chinese population for gastric cancer [37] and in Tunisian population for breast carcinoma [38]. Other studies also described association of the high producer allele in other polymorphic region of the *IL-1β* gene (-511 C/T) [39, 40] suggesting that the high secretion of IL-1β can affect individual susceptibility to the progression of these diseases [39].

Al-Tahhan et al. (2011) [40] showed that plasma *IL-1β* level is a risk factor for cervical cancer and *IL-1 β* may be involved in early step of cervical carcinogenesis and that inter-individual difference of IL-1β secretion may affect individual susceptibility to cervical cancer progression. Thus, we hypothesize that high production genotype of *IL-1β* acts like a double edge sword. At same time *IL-1β* acts protecting organism from HPV infection, but once infection being installed, high production of *IL-1β* could maintain inflammation status, contributing to cervical lesions progression and carcinogenesis.

Several studies have shown that other factor independent of HPV infection are involved in the progression of cervical lesions, as smoking [41-43]; alcohol consumption [44-46] and oral contraceptive use [43, 47, 48]. Ours results were similar for smoking and use of OCs (1.72 fold and 8.59 fold, respectively), however we did not find association for alcohol consumption (Table III).

Poppe et al (1995) [49] believed that the exposure to tobacco might affect the ability of the host to mount an effective local immune response against viral infections, as it has been shown that smoking may reduce the number of Langerhans cells and other markers of immune function. Regarding to OC use, the results could be explained taking into account that hormonal-related mechanisms may influence the progression from pre-malignant to malignant cervical lesions by promoting integration of HPV-DNA into the host genome, which results in deregulation of E6 and E7 expression [47]. Experimental studies have shown that estradiol may stimulate the transcription of HPV16 E6 and E7 in cell lines that contain integrated HPV16 [50].

Moreover, in the present study when we analyzed the combined effect of these risk factors with the presence of at least one T allele for *IL-1β* +3953 C/T polymorphism, it was observed an elevated risk to development of cervical lesions, especially for OCs, that have shown approximately 30-fold increased risk in our study population.

Based on this study we can conclude that the polymorphism *IL-1β* +3953 C/T is associated with protection against infection by HPV and with increased risk to development of cervical lesions once HPV infection are already installed.

Finally, among the host behavioral variables, a positive association was observed among smoking, and OC use with development of cervical lesions. After analyzes of combined effect between cytokine genotypes and alcohol consumption, smoking or OC use we found an increased risk for malignance.

Since we conducted our study in one population from Brazil other replicas studies are strongly envisaged to reach a consistent conclusion about the role of *IL-1β* +3953 polymorphism as marker for HPV infection and cervical lesions development.

CONFLICT OF INTEREST

No conflict of interest to declare.

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Table I. Genotypic and allelic frequencies of *IL-1β +3953* polymorphism (rs1143634) in women HPV+ and healthy controls.

	Controls (n=212)	**Cases (n=311)**	**P 95%CI OR**
GENOTYPES			
CC	88 (41.5%)	170 (54.7%)	Reference (OR=1)
CT	99 (46.7%)	134 (43.1%)	p= 0.0693; CI 0.49 - 1.01; OR 0.7
TT	25 (11.8%)	7 (2.2%)	p = <0.0001; CI 0.06 – 0.35; OR 0.14
CT + TT	124 (58.5%)	141 (45.3%)	p = 0.00311; CI 0.41 – 0.84; OR 0.59
ALLELES			
C	275 (65%)	474 (76%)	Reference (OR=1)
T	149 (35%)	148 (24%)	p = <0.0001; CI 0.44- 0.76; OR 0.58

P= p-value. 95%CI= 95% confidence interval. OR= Odds Ratio.

Table II. Genotypic and allelic frequencies of *IL-1β +3953* polymorphism (rs1143634) in women HPV+ with and without lesions.

	HPV+ without lesion (n=135)	**HPV+ with lesion (n=176)**	**P 95%CI OR**	**LSIL**	**HSIL/ICC**	**P 95% CI OR**
GENOTYPES						
CC	97 (71.8%)	73 (41.5%)	Reference (OR=1)	25 (49%)	48 (38.4%)	Reference (OR=1)
CT	35 (25.9%)	99 (56.2%)	p= <0.0001; CI 2.3 – 6.14; OR 3.76	25 (49%)	74 (59.2%)	p= 0.2653; CI 0.8 – 3; OR 1.54
TT	3 (2.2%)	4 (2.3%)	p = <0.7246; CI 0.38 – 8.16; OR 1.77	1 (2%)	3 (2.4%)	p= 0.8712; CI 0.15 – 15.8; OR 1.56
CT + TT	38 (28.1%)	103 (58.5%)	p = 0.0001; CI 2.23 – 5.82; OR 3.6	26 (51%)	77 (61.6%)	p= 0.2591; CI 0.8 – 2.8; OR 1.54
ALLELES						
C	229 (84.8%)	245 (69.6%)	Reference (OR=1)	75 (73.5%)	170 (68%)	
T	41 (15.2%)	107 (30.4%)	p = <0.0001; CI 1.63 – 3.65; OR 2.44	27 (26.5%)	80 (32%)	p= 1.3072; CI 0.78 – 2.18; OR 1.31

P= p-value. 95%CI= 95% confidence interval. OR= Odds Ratio.

Table III Association between IL-1β+3953 genotype and tobacco smoking, alcohol consumption and oral contraceptive (OC) use habits in women HPV+ with and without cervical lesion

GENOTYPES	HPV+ without lesion (n=132)	HPV+ with lesion (n=176)	P 95%CI OR
ALL			
No smoking	96 (72.7%)	107 (60.8%)	Reference (OR=1)
Smoking	36 (27.3%)	69 (39.2%)	P= 0.039; CI 1.05 – 2.8; OR 1.72
CC			
No smoking	68	51	Reference (OR=1)
Smoking	27	22	p= <0.9431; CI 0.56 – 2.12; OR 1.09
CT + TT			
Smoking	9	47	p = <0.0001; CI 3.13 – 15.5; OR 6.96
ALL			
No alcohol	71 (53.8%)	74 (40%)	Reference (OR=1)
Alcohol	61 (46.2%)	102 (60%)	p= 0.0539; CI 1.02 – 2.53; OR 1.6
CC			
No alcohol	50	33	Reference (OR=1)
Alcohol	45	40	p= <0.4245; CI 0.73 – 2.48; OR 1.35
CT + TT			
Alcohol	16	62	p = <0.0001; CI 2.9 – 11.87; OR 5.87
ALL			
No OC	113 (85.6%)	72 (40.9%)	Reference (OR=1)
OC	19 (14.4%)	104 (59.1%)	P= <0.0001; CI 4.85 – 15.21; OR 8.59
CC			
No OC	82	32	Reference (OR=1)
OC	13	41	p= <0.0001; CI 3.83 – 17.04;

			OR 1.09
CT + TT			
OC	6	63	p = <0.0001; CI 10.6 – 68.31; OR 29.9

P= p-value. 95%CI= 95% confidence interval. OR= Odds Ratio.

ANEXO A- ARTIGO PUBLICADO

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Role of Inflammasome Genetics in Susceptibility to HPV Infection and Cervical Cancer Development

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Problem: Only a small proportion of HPV+ women develop virus-associated lesions and cervical cancer, suggesting that other factors are involved in HPV+ keratinocyte transformation. Immune response plays an important role in clearing HPV infection, and host genetic variants resulting in defective immune response have been associated with virus persistence and/or cervical cancer. Considering that genetic variations in inflammasome genes were previously associated with viral infection and cancer development, the present study investigates selected single nucleotide polymorphisms (SNPs) in inflammasome genes as a possible risk factor for HPV infection susceptibility and/or for progression to cervical cancer. Patients and methods: 12 SNPs in seven inflammasome-related genes (*NLRP1, NLRP3, NLRP6, CARD8, IL1B, IL18, TNFAIP3*) were genotyped in a Brazilian HPV+ case/control cohort (n = 246/310). Multivariate analysis was performed in case/control as well as in HPV+ women stratified by the presence or severity of histologic lesion, HPV persistence, and type of virus. Results: *IL1B* rs1143643 was associated with protection against HPV infection in case/control analysis. *NLRP1* rs11651270 plays a protection role against HPV persistence and/or oncogenesis. *NLRP3* rs10754558 and *IL18* rs1834481 exert a beneficial role against HPV persistence. *NLRP3* rs10754558 variant resulted significantly associated with a lower risk to be infected with a high-risk HPV. Conclusion: Our findings for the first time demonstrated that inflammasome genetics could affect HPV/host interaction in terms of virus susceptibility as well as of virus/persistence and cervical cancer progression. ***J. Med. Virol. 88:1646–1651, 2016.*** © 2016 Wiley Periodicals, Inc.

KEY WORDS: HPV; inflammasome; NLRP3; IL-1ß; IL-18

Human papilloma virus (HPV) productively infects undifferentiated keratinocytes of cervix mucosa, however, the infection remains largely asymptomatic and it is commonly cleared within 2 years. In a small number of women, the virus cannot be eliminated, persists, and affects keratinocytes' viral detecting as well as keratinocyte-mediated innate and adaptive immune response [Karim et al., 2011].

High-risk (HR) HPVs (i.e., HPV-16 and HPV-18) are involved not only with persistent keratinocytes infection but also with cervical intraepithelial neoplastic (CIN) lesions and cervical cancer (CC), mainly through the expression of specific viral oncoproteins E6 and E7 [zur Hausen, 2002]. Only a small proportion of infected women develop virus-associated lesions and cervical cancer suggesting that other factors are involved in HPV+ keratinocyte transformation. The immune response plays an important role in clearing HPV infection, and host genetic variants resulting in defective immune response have been associated with virus persistence and/or CC [Deshpande et al., 2005; Wang et al., 2010; Zhang et al., 2014].

Within innate immune response, the role of inflammasome was recently investigated in the host/HPV interplay due to its function in antiviral and antitumoral response [largely reviewed in Man and Kanneganti, 2015]. Inflammasomes are cytoplasmic complex mounted after sensing pathogen or danger molecular patterns to activate caspase-1-mediated processing of pro-inflammatory cytokines IL-1ß and IL-18. Different pattern recognizing receptors (i.e.,

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NLRP1, NLRP3, AIM2) are able to activate inflammasome assembling in response to specific stimuli, leading to inflammation and innate immune response. Inflammasome activation is strictly regulated by endogenous host proteins (i.e., CARD8) and by a variety of transcriptional and post-transductional mechanisms [Man and Kanneganti, 2015]. Initially described in innate immune cells, inflammasomes were identified also in non-immune cells such as keratinocytes and epithelial cells, where contribute to the first line of defence against pathogens.

Discordant findings were reported about the involvement of inflammasomes in HPV infection. Reinholz et al. [2013] showed that HPV-16 activates AIM2 inflammasome with consequent secretion of both IL-1ß and IL-18 and that this activation is directly proportional to CC progression in cervical lesions. Other study showed that HPV oncoprotein E6 is able to inhibit IL-1ß secretion through a post-translational mechanism [Niebler et al., 2013].

While genetic variations in inflammasome encoding genes were associated to virus infection such as HIV-1 [Pontillo et al., 2012] and HTLV-1 [Kamada et al., 2014], they have not been yet investigated as a possible risk factor for HPV infection susceptibility and/or for progression to cervical cancer.

The present study investigates the significance of 12 selected single nucleotide polymorphisms (SNPs) in inflammasome-related genes in a Brazilian cohort of women with HPV infection (HPV+) and different stage of cervical intraepithelial neoplasia.

Five hundred fifty-six women were recruited in the metropolitan area of Recife (Northeast of Brazil) after written informed consent, as specified by the Brazilian National Council of Health (Protocol CEPSH/CPqRR/03/2008).

Three hundred ten women were negative for HPV (HPV−) at the moment of screening and were included in the study as healthy controls. Two hundred forty-six women resulted positive for HPV (HPV+).

Based on cervical biopsy results, HPV+ women were classified as without histologic lesion (w/o L; n = 63), with mild cervical intraepithelial neoplasia (CIN-I; n = 79), moderate cervical intraepithelial neoplasia (CIN-II; n = 38), severe cervical intraepithelial neoplasia (CIN-III; n = 47), or Cervical Cancer (CC; n = 17). In some analysis, moderate and severe CIN were grouped in high-grade squamous intraepithelial lesion (HSIL), while mild CIN was called low-grade squamous intraepithelial lesion (LSIL) according to the Bethesda System [Wright, 2006].

HPV+ women were also genotyped for HPV type-specific E7 to determine HR and low-risk (LR) virus according to [Chagas et al., 2015].

Table I summarizes main data of the case/control cohort.

Twelve single-SNPs in seven inflammasome genes (rs12150220 and rs11651270 in *NLRP1*, rs35829419 and rs10754558 in *NLRP3*, rs2043211 and rs6509365 in *CARD8*, rs1143643 and rs1143629 in *IL1B*, rs5744256 and rs1834481 in *IL18*, rs4758635 in *NLRP6*, rs6920220 in *TNFAIP3*) were selected based on previously reported association with viral diseases [Pontillo et al., 2012, 2013; Motavaf et al., 2014; Kamada et al., 2014] or cancer development [Verma et al., 2012; Palmieri et al., 2008; Chen et al., 2011; Ungerbäck et al., 2012].

TABLE I. Main Characteristics of Brazilian HPV Case/Control Cohort

Characteristics	HPV+ (n = 246)	HPV− (n = 310)
Age (years)	35 ± 11.42	35 ± 10.0
Partners (n)	2.3 ± 1.9	1.9 ± 1.8
Histologic forms		
without lesion	63 (0.26)	–
CIN-I	79 (0.32)	–
CIN-II	38 (0.15)	–
CIN-III	47 (0.19)	–
Cervical cancer	17 (0.07)	–
NC	2 (0.01)	–
Cervical lesions		
HSIL (CIN-II + CIN-III)	85 (0.35)	–
LSIL (CIN-I)	79 (0.32)	–
Viral sub-types		
LR-HPV	35 (0.14)	–
HR-HPV	211 (0.86)	–
HPV-16	132 (0.54)	–

CIN, cervical intraepithelial neoplasia; NC, not classified; HSIL, high-grade squamous intraepithelial lesion; LSIL, low-grade squamous intraepithelial lesion; LR-HPV, low-risk HPV; HR-HPV, high-risk HPV.
Values are reported as mean ± SD, ratio or count (frequency).

Genotyping was performed using TaqMan® SNP assays and StepOne Plus equipment (Applied Biosystems, Waltham, MA). Allelic discrimination was performed using the StepOne Plus software. R software (www.r-project.org) was used for multivariate analysis (age and number of partners were considered as independent variables), inheritance modeling, and epistasis analysis (package "SNPassoc" version 1.5–2). According to recent reports, the Brazilian population [Pena et al., 2011], and in particular Recife [Coelho et al., 2015], has a low level of genetic substructure and for this reason we did not included ethnicity in multivariate analysis.

Haploview software was used to investigate the linkage disequilibrium (LD) pattern and for deriving the haplotypes.

All the SNPs frequencies were found to be in Hardy–Weinberg equilibrium in our cohort.

No association was detected between inflammasome SNPs and the susceptibility to HPV (case/control study) with the exception of the *IL1B* rs1143643_G>A (Supplementary File 1). *IL1B* rs1143643 genotypes carrying the minor A allele were significantly less frequent in HPV+ (0.06) than in HPV− (0.09) women, and behaved according to a dominant model of inheritance (G/A + A/A vs. G/G; $P = 0.04$, OR = 0.71) (Table II), suggesting that *IL1B* rs1143643 confers protection against HPV infection.

TABLE II. Association Results for Case/Control Analysis

Genotypes	HPV+ (n = 246)	HPV− (n = 310)	*P*	OR (95%CI)	P_{adj}	OR_{adj} (95%CI)
G/G	136 (0.58)	152 (0.49)	0.04	Ref.	0.04	Ref.
G/A	85 (0.36)	129 (0.42)		0.71 (0.50–1.0)		0.71 (0.50–0.99)
A/A	14 (0.06)	27 (0.09)				

Association results for *IL1B* rs1143643 according to GLM analysis in women with (HPV+) and without (HPV+) HPV infection. *P*-value unadjusted (p) and adjusted for age and number of partners (padj) are reported as well as odds ratio (OR) and confidence interval (95%CI) toward the reference genotype (Ref). Best-fit inheritance model (according to Akaike Index Criterium) is reported.

Although *IL1B* rs1143643 is a tag SNP without an established functional effect, we can speculate that this variation could affect IL-1ß production. Besides its role in inflammation, IL-1ß contributes to T helper (Th) polarization, in particular toward a Th17 profile. Recent findings reported the negative role of Th17 and IL-17 in anti-HPV immune response [Gosmann et al., 2014], leading us to hypothesize that a lower production of IL-1ß could be related to a less Th17 polarization and consequently to a better anti-viral response.

In our cohort, linkage disequilibrium was observed only for *CARD8* rs2043211 and rs6509365 (LOD/D′ = 0.97) (Supplementary File 2). The resulting three haplotypes (A-A, T-G, A-G), however, were similarly distributed in HPV+ and HPV− women ($P > 0.05$).

Then we analyzed SNPs significance in relation to the presence or severity of histologic lesion and cancer ("w/o L + LSIL" vs. "HSIL + CC"), HPV persistence ("w/o L" vs. "LSIL/CIN-I"), type of virus ("HR-HPV" vs. "LR-HPV").

Stratifying HPV+ women by progression to cervical cancer ("w/o L + LSIL" vs. "HSIL + CC") revealed a significantly reduced risk for HPV oncogenesis in the presence of *NLRP1* rs11651270_T>C (Table III). Minor C allele was less frequent in HPV+ women with higher grade of intraepithelial lesions and CC ("HSIL + CC") when compared to HPV+ women without lesion or with lower grade of lesion ("w/o L + LSIL") (0.16 vs. 0.27; $p_{adj} = 0.003$; $OR_{adj} = 0.43$).

This *NLRP1* polymorphism was previously associated to an augmented IL-1ß and IL-18 processing [Levandowski et al., 2013] and, interestingly, to breast cancer in a GWAS study [Gao et al., 2012], suggesting that the NLRP1-inflammasome activation could be beneficial to the host against cancer development. Of note, NLRP1 interacts with Bcl-2 and Bcl-X and it is involved in the apoptotic pathway [Bruey et al., 2007; Faustin et al., 2009], which is affected in transformed HPV+ keratinocytes [Alibek et al., 2014].

Un-expectantly, two inflammasome genes involved in cancer development, *NLRP6* [Chen et al., 2011] and *TNFAIP3* [Ungerbäck et al., 2012], did not alter the risk to develop HSIL and CC, at least in our cohort (Supplementary File 3).

When stratifying according to HPV persistence ("w/o L" vs. "LSIL/CIN-I"), a significant association was observed for the *NLRP3* rs10754558_C>G and *IL18* rs1834481_C>G variants (Supplementary File 4).

Genotypes carrying rs10754558 minor G allele were significantly less frequent in HPV+ women with CIN-I (0.45) than in HPV+ women w/o L (0.67) indicating a protection role of this polymorphism toward virus persistence/ ($p_{adj} = 0.008$; $OR_{adj} = 0.40$ according to a dominant model of inheritance) (Table III).

Our previously published data reported a similar association with HIV-1 [Pontillo et al., 2012] and HTLV-1 infections [Kamada et al., 2014], emphasizing that the NLRP3-inflammasome plays a key role in innate immune anti-viral response and possibly in subversion of virus persistence and viral clearance.

In a similar way, *IL18* rs1834481 minor G allele was more frequent in HPV+ women w/o L (0.28) compared to CIN-I (0.26), suggesting a beneficial role of this variant against HPV persistence especially when the analysis was adjusted for women age and number of partners ($p_{adj} = 0.018$, according to a dominant model of inheritance).

This intronic variant is known to affect IL-18 plasma level and it was previously associated with the progression toward several types of cancer [Palmieri et al., 2008; Haghshenas et al., 2009; Khalili-Azad et al., 2009].

Of note, *IL18* promoter polymorphisms, that also affect IL-18 plasma level, play a protective role against the development of chronic HBV infection [Motavaf et al., 2014], suggesting a common mechanism against oncogenic viruses.

Upon stratification by oncogenic risk level of HPV ("HR-HPV" vs. "LR-HPV"), a diminished risk to be infected with a HR-HPV was seen for women carrying the *NLRP3* rs10754558 variant ($p_{adj} = 0.005$; $OR_{adj} = 0.32$, according to a dominant model of inheritance) (Table III), highlighting once more that NLRP3-inflammasome activation is important in antiviral immune response. The others SNPs did not appeared associated to virus type (Supplementary File 5).

Finally, considering that inflammasome is a multiprotein complex and that all studied molecules interact each other to activate IL-1ß and IL-18 processing, we hypothesized that SNPs in different genes could display a combined effect and influence susceptibility to HPV infection and/or the development of oncogenic transformation. The gene–gene interaction analysis was performed in case/control as well as in HPV+ subgroups comparisons (complete results are graphically showed in Supplementary File 6). Statistically significant results are reported in Table IV

In case/control study, *NLRP3* rs10754558 and *NLRP1* rs12150220 significantly interact according to

TABLE III. Association Results for HPV+ Women Sub-Classified for Intraepithelial Lesion or Oncogenic Virus Type

	Genotypes			*P*	OR (95%CI)	p_{adj}	OR_{adj} (95%CI)
			Histologic subgroup analysis				
		HSIL + CC (n = 140)	w/o L + LSIL (n = 101)				
NLRP1 rs11651270	T/T	51 (0.36)	20 (0.20)	0.004	Ref.	0.003	Ref.
	T/C	67 (0.48)	53 (0.53)		0.43 (0.24–0.78)		0.41 (0.22–0.75)
	C/C	22 (0.16)	28 (0.27)				
			Predisposition to latency				
		CIN-I (n = 79)	w/o L (n = 63)				
NLRP3 rs10754558	C/C	43 (0.55)	20 (0.33)	0.008	Ref	0.008	Ref
	C/G	27 (0.35)	33 (0.54)		0.40 (0.20–0.80)		0.40 (0.20–0.80)
	G/G	8 (0.10)	8 (0.13)				
IL18 rs1834481	C/C	55 (0.74)	39 (0.72)	0.07		0.018	
	C/G	19 (0.26)	12 (0.22)				
	G/G	0 (0.0)	3 (0.06)				
			Predisposition to HR-HPV infection				
		HR-HPV (n = 211)	LR-HPV (n = 35)				
NLRP3 rs10754558	C/C	103 (0.49)	8 (0.24)	0.005	Ref	0.004	Ref
	C/G	85 (0.40)	22 (0.65)		0.32 (1.34–7.16)		0.31 (1.36–7.33)
	G/G	23 (0.11)	4 (0.11)				

HSIL, high-risk intraepithelial lesions; CC, cervical cancer; w/o L, without intraepithelial lesions; LSIL, low-risk intraepithelial lesions; CIN-1, cervical intraepithelial neoplasia grade 1; HR, high risk; LR, low risk.

Significant association results for HPV+ women grouped based in histologic lesions (without lesion or with low grade histologic lesion vs. high grade histologic lesion or cervical cancer), predisposition to latency (without lesion vs. first grade of cervical intraepithelial neoplasia), HPV type (high-risk vs. low-risk virus) are reported according to GLM analysis. *P*-value unadjusted (p) and adjusted for age and number of partners (p_{adj}) are reported as well as odds ratio (OR) and confidence interval (95%CI) toward the reference genotype (Ref.). Best-fit inheritance model (according to Akaike Index Criterium) is dominant for all the reported SNPs.

TABLE IV. Gene–Gene Interaction Analysis

Comparison	Genes (SNPs)	p_{adj}
HPV+ versus HPV−	*NLRP1* (rs12150220)–*NLRP3* (rs10754558) (c)	0.005
	NLRP3 (rs35829419)–*CARD8* (rs6509365) (r)	0.008
w/o L + LSIL versus HSIL + CC	*NLRP3* (rs10754558)–*IL1B* (rs1143643) (r)	0.004
	NLRP3 (10754558)–*IL18* (rs1834481) (d)	0.006
	NLRP3 (rs10754558)–*CARD8* (rs2043211) (d)	0.004
HR-HPV versus LR-HPV	*NLRP3* (rs35829419)–*IL18* (rs5744256) (r)	0.005
	NLRP3 (rs35829419)–*IL18* (rs1834481) (r)	0.005

a co-dominant model of inheritance ($P = 0.005$). Taking in account severity of cervical lesion and progression to CC ("w/o L + LSIL" vs. "HSIL + CC"), *NLRP3* rs10754558 significantly interacts with *IL1B* rs1143643 and *IL18* rs1834481 ($P = 0.004$ and $P = 0.005$, respectively). Moreover, *NLRP3* rs35829419 and *CARD8* rs6509365 also interact ($P = 0.008$). A combined effect of *NLRP3* rs10754558 with *CARD8* rs2043211 ($P = 0.004$) and of *NLRP3* rs35829419 with the two SNPs in *IL18* ($P = 0.005$) was found comparing HR-HPV and LR-HPV women.

Epistasis analysis emphasized that NLRP3′ variants together with polymorphisms in other inflammasome-related genes, modulating the rate of inflammasome activation and/or the preferential processing of IL-1ß or IL-18, could affect the outcome of HPV infection or cervical cancer progression.

In conclusion, our findings, for the first time to our knowledge, demonstrated that inflammasome genetics could affect HPV/host interaction in term of virus susceptibility as well as of virus/persistence and cervical cancer progression. Further investigations are needed to elucidate the interplay between inflammasomes and HPV.

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ANEXO B – ARTIGO PUBLICADO

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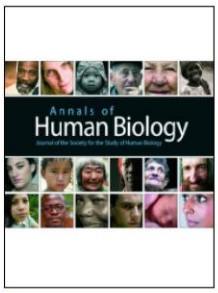

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RESEARCH PAPER

Tumor necrosis factor (TNF) alpha and interleukin (IL) 18 genes polymorphisms are correlated with susceptibility to HPV infection in patients with and without cervical intraepithelial lesion

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Abstract

Background: The Human Papillomavirus (HPV) predisposes 500 000 women to cervical cancer. Host genetic background may facilitate virus persistence in the uterine cervix. Polymorphisms in regulatory and coding regions of cytokine genes have been associated with susceptibility to some human diseases.

Aim: This study aims at investigating whether *TNFA* -308 G/A and *IL18* -137 G/C and -607 C/A polymorphisms are associated with susceptibility to HPV infection/progression to high-grade squamous intraepithelial lesion (HSIL).

Subjects and methods: One hundred and twenty-two HPV infected and 132 HPV negative women (the latter used as healthy controls) were analysed. *TNFA* -308 G/A and *IL18* (-137G/C and -607 C/A) polymorphisms were analysed using specific sequence polymorphism PCR (SSP-PCR). Univariate statistical analysis and a logistic regression were performed.

Results: The *TNFA* -308A allele was associated with susceptibility to HPV infection ($p = 0.0008$), while the *IL18* -607A allele conferred protection against HPV infection ($p = 0.0043$). *TNFA* -308 G/A and *IL18* (-137G/C and -607 C/A) polymorphisms were not associated with development of cervical lesions ($p > 0.05$). An association was also observed between smoking and susceptibility to the development of HSIL.

Conclusion: The findings suggest an association between two *TNFA SNPs* and susceptibility to HPV infection in women from Northeast Brazil. The results need to be functionally validated and replicated in other populations with different ethnic backgrounds.

Keywords

Cytokine, invasive cervical cancer, single nucleotide polymorphism

History

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Background

Cervical cancer is the second most common malignant tumor in the world after breast cancer, affecting ~530 000 new women per year, and approximately half of them will die as a consequence of the disease. Developing countries have 80% of the new cases, making this cancer the most incident in women. Cancer caused by human papillomavirus (HPV) infections represents nearly 5% of all types of cancers (Parkin et al., 2005; WHO, 2013).

The HPV, *Papillomaviridae* family, is the main causal aetiological agent responsible for the development of 99.7% of worldwide cervical cancer cases. The persistence of HPV infection, especially the high-risk oncogenic types, can cause epithelial alterations, called Cervical Intraepithelial Neoplasm (CIN) or Squamous Intraepithelial Lesions (SIL) (Doorbar, 2006; Lizano et al., 2009; Lowy & Howley, 2001; Mammas et al., 2014; Tommasino, 2014). In Brazil, a 2013 prospective study from the National Institute of Cancer reported 15 590 new cases, resulting in an estimated risk of 15 cases per 100 000 women (BRASIL. INCA, 2014).

Besides HPV, environmental (diet, use of oral contraceptives, tobacco use, multiple sexual partners) and genetic factors also influence susceptibility to cervical cancer development (Johnson & Todd, 2000; Santos et al., 2005; Ylitalo et al., 2000).

Among the genetic factors that could be involved in the susceptibility to HPV infection are the cytokines production levels in response to microbe stimulation, which have been reported to be genotype-dependent (von Linsingen et al., 2009). Two of them, namely tumour necrosis factor-alpha (TNF-α) and interleukin 18 (IL-18), have been considered in

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this study. Our hypothesis is that polymorphisms in these genes could influence the susceptibility to HPV infection or HPV lesion progression.

The tumor necrosis factor-alpha (TNF-α) is a pro-inflammatory cytokine, mainly released by stimulated monocytes and macrophages. The 3kb *TNFA* gene is localized within the major histocompatibility complex locus at chromosome 6 (p21.3) (Duarte et al., 2005; Kirkpatrick et al., 2004) and consists of four exons (Laing et al., 2001). TNF-α has large biologic effects, including protection against infections, surveillance against tumours and stimulation of inflammatory responses (Laing et al., 2001), thus fulfilling an essential role in the immune system (Barth et al., 1996).

TNFA has many polymorphisms, with single nucleotide polymorphisms (SNPs) in the promoter region at positions -308 and -238, being the most commonly studied (Elahi et al., 2009; Kroeger et al., 1997; Wilson et al., 1997). The polymorphism at position -308 (G → A) of ***TNFA*** is already known to affect gene expression and is involved in the regulation of its transcription (Bouma et al., 1996; Fernandes et al., 2008; Wilson et al., 1997). Cabrera et al. (1995) showed that cells carrying the A allele have 6–9-fold higher transcription levels when compared to those carrying the G allele. Other *in vitro* studies have reported that the G allele is associated with a reduced production of TNF-α, while individuals with A allele produce more TNF-α (Hajeer & Hutchinson, 2001; Haukim et al., 2002; Kroeger et al., 1997; Wilson et al., 1997).

Interleukin 18 (IL-18) plays a primordial role in the immune response, being responsible for the T helper cell differentiation and activation into sub-groups, according to the cytokines profile (Nakanishi et al., 2001). IL-18 induces the differentiation and proliferation of T helper cells in the presence of interleukin 12, stimulating the production of interferon and tumour necrosis factor (TNF) in T lymphocytes and natural killer cells (Esfandiari et al., 2001; Gracie et al., 2003; Nakanishi et al., 2001). Five SNPs in the promoter region of *IL18* gene (11q22.2–q22.3) have been described: -656 G/T, -607 C/A, -137 G/C, 113 G/T and 127 C/T (Giedraitis et al., 2001). However, only the SNPs at positions -137 G/C and -607 C/A were confirmed as having an impact on the activity of *IL18* gene in previous studies (Piskin et al., 2013).

The role of *TNFA* -308 G/A and *IL18* (-137G/C and -607 C/A) polymorphisms on the susceptibility to many infectious diseases has been well documented (Castelar et al., 2010; Corchado et al., 2013; Yue et al., 2013), but the functional importance about these polymorphisms is still debated.

Therefore, this study aims at analysing the possible association of *TNFA* (-308 G/A) and *IL18* (-137G/C and -607 C/A) promoter SNPs with susceptibility to HPV infection as well as to (pre) neoplastic cervical disease in a population from Northeast Brazil.

Methods

Design and study site

A cross-sectional study was performed aimed at analysing ***TNFA*** and *IL18* gene polymorphisms in HPV-infected women with cervical lesions and invasive cervical cancer (ICC). Women were recruited at the Lower Genital Tract Pathology Clinic at the Women's Healthcare Centre of the Prof. Fernando Figueira Institute of Integrated Medicine (Recife, Brazil) between December 2008 and December 2009.

The laboratorial analyses were conducted at the Laboratory of Genetics, Biochemistry and DNA sequencing, Professor Tania Falcão of the Universidade Federal Rural de Pernambuco (Recife, Brazil). The 'Universidade Federal Rural de Pernambuco' Research Ethics Committee approved the study and all patients and controls gave written consent (protocol number 355/08).

Study subjects

We studied 122 women from Recife metropolitan region (Pernambuco, Northeast Brazil), with ages between 16–75 years, mean age 32.9 ± 10.8 years, presenting either low-grade squamous intraepithelial lesion (LSIL) or high-grade squamous intraepithelial lesion (HSIL). The onset of Invasive Cervical Cancer (ICC) is defined when these lesions infiltrate to others tissues. Patients were included in the study if there was no discrepancy between their cytological abnormality and the histological diagnosis made at the first visit. All patients were initially assessed by colposcopy and subsequently cervical smears were collected. Histological diagnosis was made according to Solomon et al. (2002) and Associação Brasileira de Ginetoscopia (2002). Patients were also stratified according to smoking and alcohol consumption.

One hundred and thirty-two unrelated women volunteers from Recife metropolitan region, aged between 14–70 years (mean age 37.7 ± 10 years), with no history of lesions or neoplastic disease as evaluated by the physician were enrolled as controls and written informed consent was obtained.

A questionnaire considering social and demographic features such as age, level of instruction, age of the first sexual relationship, number of partners and sexual behaviour, as well as environmental factors (i.e. smoking and alcohol consumption) was given to both patients and controls, subsequently matched for these characteristics.

Our patients and controls were evaluated for ethnicity using genetic ancestry markers (AIMs), since commonly used self-reported skin colour classification in questionnaires is a quite poor marker for ethnicity. The genetic ancestry of both patients and controls was assessed using the criteria of Kosoy et al. (2009) with an 'in house' modification to reduce genotyping costs consisting of the use of the following 12 SNPs: rs4908343, rs7554936, rs6548616, rs7657799, rs10007810, rs6451722, rs1040045, rs10108270, rs772262, rs9530435, rs11652805, rs4891825.

Sample collection and genomic DNA extraction

The samples were collected by scraping the cervical region with a cytobrush. The brushes were immediately placed in 1.5 mL TE maintenance buffer (10 mM Tris-HCl and 1 mM EDTA pH 8.0). The samples were processed for DNA extraction immediately after transport to the laboratory. The extraction was performed with Nucleospin Tissue kit (Macherey-Nagel, Belo Horizonte, MG, Brazil) following the manufacturer's instructions. The DNA solution was then stored at −20 °C until use.

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HPV detection and typing

All samples were tested for HPV presence using MY09/11, GP05+ and GP06+ consensus primers following PCR protocols published elsewhere (De Roda Husman et al., 1995; Manos et al., 1989). Amplification of human β-globin gene segment was used as an internal control for DNA quality and samples negative for this assay were excluded from analysis. High-risk HPV 16 and 18 genotyping was performed using specific primers following protocols published elsewhere (Karlsen et al., 1996). All primers used in this study are listed in Table 1.

Sample sequencing

The HPV positive samples, which tested negative for HPV 16 and 18 sub-types, were submitted to sequencing reactions to detect whether other HPV strains were present. This was done using the kit DyEnamic ET Dye Terminator Cycle sequencing kit (GE Healthcare) following the manufacturer's instructions on the automated DNA sequencer MegaBACE 1000 DNA (Sigma-Aldrich Brasil Ltda., Sao Paulo, Brazil).

Results were then compared with HPV genotypes already available through the online platform BLASTn of the National Center for Biotechnology Information (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

TNFA and *IL18* polymorphisms analysis

The SNPs were detected using specific sequence polymorphism polymerase chain reaction (SSP-PCR). Briefly, the ***TNFA*** -308 G/A (rs1800629) polymorphism genotyped was performed as described by Perrey et al. (1999), while *IL18* -137 G/C (rs187238) polymorphism was detected as previously described by Arimitsu et al. (2006). The -607 C/A (rs1946518) polymorphism was genotyped using the protocol of Yu et al. (2009).

The amplification reaction for analysis of ***TNFA*** polymorphism was performed in a final volume of 15 µl. The reaction mixture contained 200 ng of DNA from vaginal cytobrush specimens, 1X Buffer of Taq Platinum® (Invitrogen Brasil Ltda., Sao Paulo, SP, Brazil), 200 µM of deoxynucleotide-triphosphates, 2.5 mM $MgCl_2$, 1U DNA Taq Platinum DNA polymerase (Invitrogen Brasil Ltda., Sao Paulo, SP, Brazil) and 1 µM of each primer (common and specific primer; Table 1). Thermal cycling conditions were the same as stated in the literature (Arimitsu et al., 2006; Perrey et al., 1999; Yu et al., 2009).

To confirm PCR-SSP results, 40 samples randomly chosen were genotyped by direct sequencing and 100% concordance was found.

Statistical analysis

Univariate statistical analysis was performed using the BioEstat 5.0 software. The study was cross-sectional, with independent samples consisting of nominal data (genotypes). The influence of each polymorphism on the risk for development of (pre) neoplastic cervical disease was estimated by odds ratio (OR) and a 95% confidence interval (CI).

Allele frequencies were estimated by direct counting. Comparison between genotypic frequencies of patients and control groups was performed by χ^2 test and Fisher's exact test was used to compare the allele frequencies in contingency tables.

For identification of relevant risk factors, a logistic regression analysis was carried out (comparing HPV-positive women with history of lesions with HPV-positive women with no history of lesions or cervical cancer). This modelled the influence of genetic polymorphisms, HPV 16 single infection or multiple HPV strains co-infection, smoking and alcohol consumption on the risk of developing HSIL. The OR and their respective 95% CI were determined. The R

Table 1. Sequence of primers for PCR quality control check, HPV detection and typing and for genotyping of polymorphisms in cytokines genes.

Type	Primer	Primer sequence	Product length (bp)
Human β-globin	PC04	5′-GAA GAG CCA AAG GAC AGG TAC-3′	268
	GH20	5′-GAA GAG CCA AGG ACA GGT AC-3′	
MY[a]	MY09	5′-CGT CCM ARR GGA WAC TGA TC-3′	450
	MY11	5′-GCM CAG GGW CAT AAY AAT GG-3′	
GP+[b]	GP05+	5′-TTT GTT ACT GTG GTA GAT ACT AC-3′	150
	GP06+	5′-GAA AAA TAA ACT GTA AAT CAT ATT C-3′	
HPV16[c]	PR3	5′-GTC AAA AGC CAC TGT GTC CT-3′	499
	PR4	5′-CCA TCC ATT ACA TCC CGT AC-3′	
HPV18[c]	PR1	5′-CCG AGC ACG ACA GGA ACG ACT-3′	172
	PR2	5′-TCG TTT TCT TCC TCT GAG TCG CTT-3′	
TNF α (-308 G/A)[d]	G allele	5′-ATA GGT TTT GAG GGG CAT GG-3′	233
	A allele	5′-AA T AGG TTT TGA GGG GCA TGA-3′	
	generic	5′-TCT CGG TTT CTT CTC CAT CG-3′	
IL18 (-137 G/C)[e]	G allele	5′-CCC CAA CTT TTA CGG AAG AAA AG-3′	261
	C allele	5′-CCC CAA CTT TTA CGG AAG AAA AC-3′	
	generic	5′-AGG AGG GCA AAA TGC ACT GG-3′	
IL18 (-607 C/A)[f]	C allele	5′-TTC TGC ATC TTT ACA GCT GGA G-3′	301
	A allele	5′-CAC GGA TAC CAT CAT TAG AAT TTT ATT-3′	
	generic	5′-TTC TGC ATC ITT ACA GCT GGA-3′	

[a]Manos et al. (1989); [b]De Roda Husman et al. (1995); [c]Karlsen et al. (1996); [d]Perrey et al. (1999); [e]Arimitsu et al. (2006); [f]Yu et al. (2009).

software version 3.0.2 (http://www.R-project.org/) was used to perform the regression analysis. Power analysis was performed through G*Power software version 3.1.9.2 (Faul et al., 2007).

All p values under or equal to 0.05 were considered statistically significant.

Results

All controls were tested for the presence of HPV and the result was negative, confirming the absence of HPV infection. The HPV infected group samples with detected lesions were stratified according to the degree of cervical intra-epithelial neoplasia: 20.49% (25/122) of patients had LSIL, 70.5% (86/122) had HSIL and 9.02% (11/122) had ICC.

The distribution of HPV genotypes and co-infections is shown in Table 2. The prevalence of single infection was 79.5% (97/122) and multiple infections represented 20.49% (25/122) of all cases. Among the patients with single infection, 49.2% (60/122) were infected with HPV 16, 15.6% (19/122) with HPV 18, 13.9% (17/122) with other high-risk (HR-HPV) types (31, 33, 56, 58, 67 and 83) and 1.6% (2/122) were diagnosed as low-risk (LR-HPV). Considering multiple infections, HPV16/18 co-infection was detected in 16.4% (20/122) of patients; and 4.1% (5/122) of patients were diagnosed with simultaneous co-infections between HPV16, 18 and other HPV types.

Two risk factors, namely smoking and alcohol consumption, were evaluated for association with squamous intra-epithelial lesion degree and ICC development, 63.9% (78/122) reported alcohol consumption and 38.5% (47/122) reported smoking. No significant differences were observed between patients and control groups when we evaluated smoking and drinking status with progression of HPV-related cervical lesions during univariate analysis ($p = 0.2035$ and $p = 0.7217$, respectively). In addition, both cases and controls presented a similar ethnic background: 58% European, 27% African and 15% Amerindian; so we assumed that the SNPs' frequencies were not related to any specific particular ethnic group. Therefore, any association found is supposed not to be a consequence of population structure, but rather a real effect on disease.

Table 3 shows the distribution of genotypes and allele frequencies of ***TNFA*** -308 G/A and *IL18* (-137 G/C and -607 C/A) polymorphisms in patients and healthy controls and their genetic association tests and achieved power.

Table 2. Prevalence of HPV sub-types, overall and specific infection rate and co-infections occurrence in the study population.

Type of infection	$n = 122$ (%)*
Overall single infection	97 (79.5)
HPV 16	60 (49.2)
HPV 18	19 (15.6)
Single infection – other HR-HPV (31,33,56,58,67 or 83)	17 (13.9)
Single infection – other LR-HPV (70)	1 (0.82)
Overall multiple infections	25 (20.5)
Co-infection HPV16/18	20 (16.4)
Co-infection HR + LR (6/18 and 18/61)	2 (1.6)
Co-infection HR (16/18/56, 18/31 and 31/67)	3 (2.5)

HR-HPV, high-risk HPV; LR-HPV, low-risk HPV.
*All percentages are relative to total sample size ($n = 122$).

When considering *IL18* polymorphisms, we observed a reduced risk of HPV infection in patients carrying the -607 AA genotype ($p = 0.0043$), but this genotype was not associated with progression to higher grades of cervical lesions ($p = 0.4038$). Furthermore, no significant association was found between *IL18* -137 polymorphism allele frequencies and susceptibility to HPV infection (OR = 0.71, 95% CI = 0.48–1.04; $p = 0.0757$) or with pre-disposition to HPV-related cervical lesions development (OR = 0.59, 95% CI = 0.30–1.15; $p = 0.1176$). Similarly, *IL18* -137 genotypes were not associated with HPV infection ($p = 0.1382$) and the presence of cervical lesions ($p = 0.1445$).

The ***TNFA*** -308 G allele was significantly associated with increased risk of HPV infection (OR = 1.91 95% CI = 1.31–2.78; $p = 0.0008$), but not with progression of HPV-related cervical lesions (OR = 1.20, 95% CI = 0.63–2.28; $p = 0.5872$). The same was observed for the genotypes ($p = 0.0008$ for HPV infection association test and $p = 0.6174$ for cervical lesion association test).

Finally, with the aim of evaluating the possible confounder effects represented by smoking or alcohol consumption on susceptibility to HPV infection or HSIL development we performed a logistic regression analysis. We found an association of smoking with the susceptibility to HSIL in the study population, as indicated in Table 4 (OR = 3.569; 95% CI = 1.0994–11.5864; $p = 0.03$).

Discussion

In our study group, high risk HPV 16 and 18 types were the most frequent: this result agrees with previous findings reported for the Brazilian population (Lippman et al., 2010; Oliveira-Silva et al., 2011; Tavares et al., 2014). HPV 16 is the most frequent type in Brazil, but regional variations have been observed (Ayres & Silva, 2010; Fernandes et al., 2013; Ribeiro et al., 2011).

We tested the hypothesis that functional polymorphisms in genes encoding cytokines might influence the susceptibility to HPV infection and/or the susceptibility to progression of HPV-related cervical lesions. Our data support the hypothesis that ***TNFA*** -308 G/G genotype and -308 G allele might be risk factors to HPV infection (OR = 2.46; $p = 0.0008$; OR = 1.91; $p = 0.0008$, respectively). On the other hand, *IL18* -607 A/A genotype was associated with protection against HPV infection (OR = 0.68, $p = 0.0043$). No significant association was found for *IL18* gene -137G/C polymorphism and susceptibility to HPV infection. Furthermore, no association was observed for these polymorphisms in relation to progression of HPV-related cervical lesions.

Kirkpatrick et al. (2004) studied British patients exhibiting cervical lesions and suggested that ***TNFA*** -308 GG genotype, present in 95% of patients with LSIL, was conferring protection against severe cervical disease. Fernandes et al. (2008) studied 42 European descendant women from São Paulo, Brazil, aged between 16–46 years, all presenting cervical lesions, and they did not find significant differences in the distribution of ***TNFA*** -308 G/A between patients and controls.

In our study, the frequency of ***TNFA*** -308 GG genotype in patients with LSIL (32%) and HSIL (30%) was not significantly different ($p > 0.05$, Table 2).

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Govan et al. (2006) did not find an association between ***TNFA*** -308 G/A polymorphism and cervical cancer in patients from South Africa. Stanczuk et al. (2003) reported that the *TNFA* -308 G/G genotype was present in 72% of cervical cancer patients infected by HPV in a study population from Zimbabwe. Duarte et al. (2005) observed that the ***TNFA*** -308 AA genotype was more frequent in Portuguese patients with cervical cancer than in controls (3.6% and 1.6%, respectively), but these results were not statistically significant.

Our results showed a higher percentage of ***TNFA*** -308 AA homozygous genotype in HSIL patients (10%) when compared to LSIL (4%) and healthy controls (4%) (Table 2); however, this difference was not significant ($p > 0.05$).

If we consider the role of pro-inflammatory cytokine TNF-α in HPV pathogenesis, we have to bear in mind that it has been described as being involved in the control of HPV infection with direct and indirect actions. There is evidence that HPV16 infected cervical cell lines *in vitro* produce higher levels of TNF-α in an *in vitro* model when compared to HPV uninfected cells (Bequet-Romero & Lopez-Ocejo, 2000). Moreover, increased TNF-α levels have been reported in cervical cancer (Tjiong et al., 2001).

TNF-α has been hypothesized to be able to directly fight HPV infection, inducing apoptosis in HPV-infected cells as well as in cervical cancer cells (Basile et al., 2001). Another mechanism possibly used by TNF-α to prevent HPV replication and arrest its spread is the activation of the inflammatory response, increasing the expression of chemokines and molecules involved in vascular adhesion. Finally, TNF-α can indirectly promote HLA class I up-regulation, with major expression of non-professional antigen presenting cells capable of tackling HPV (Hallermalm et al., 2001).

Thus, having found in our study an association between *TNFA* -308 G/G genotype/G allele and increased susceptibility to HPV infection, we can hypothesize that this genetic variant, already known to reduce production of TNF-α, could contribute to the lack of TNF-α mediated apoptosis, low inflammatory stimuli and reduced presence of HLA class I antigen presenting molecules. Since they all act to fight HPV infection, their reduced levels would favour the spread of the virus. However, we have not found any association with HPV related lesions, as expected by the genetically driven reduced levels of TNF-α. Therefore, more studies should be conducted to better address this issue.

We also report the association of *IL18* promoter gene polymorphisms -607 C/A, with protection against HPV infection. The -607 AA genotype confers protection against HPV-infection, but it does not affect the evolution of cervical lesions.

IL-18 is an inflammatory cytokine able to induce IFN-γ production in the course of infection and plays a pivotal role in the activation of the immune system against viruses such as

Table 4. A logistic regression analysis modelling risk to HSIL development, involving *IL18* and *TNF-α* polymorphisms, HPV infection status, smoking and alcohol consumption.

Covariates	OR	95% CI	*p* Value
IL-18 -137 G/G	reference		
IL-18 -137 G/C	0.6316	0.2253–1.7701	0.1239
IL-18 -137 C/C	0.2187	0.0315–1.5163	0.3823
IL-18 -607 C/C	reference		
IL-18 -607 C/A	0.4976	0.1350–1.8337	0.2943
IL-18 -607 A/A	1.6970	0.5336–5.3972	0.3703
TNF -308 G/G	reference		
TNF -308 G/A	1.4545	0.4953–4.2714	0.4954
TNF -308 A/A	3.1015	0.3336–28.8363	0.3198
Multiple HPV-infection	1.0293	0.2579–4.1073	0.9674
HPV-16 single-infection	0.7230	0.2366–2.2091	0.5693
Smoking	3.5690	1.0994–11.5864	0.0342*
Alcohol consumption	0.5924	0.2157–1.6264	0.3096

*Significant *p*-value.

Table 3. Allele and genotype frequencies of *IL18* -137 G/C (rs187238), *IL18* -607 C/A (rs1946518) and *TNFA* -308 (rs1800629) polymorphisms among women infected with HPV and healthy controls from Pernambuco state (Northeast Brazil).

	HPV status					Cervical lesions status				
SNP	HC $n = 132$ (%)	HPV+ $n = 122$ (%)	OR (95% CI)	*p* Value	Power	LSIL $n = 25$ (%)	HSIL and ICC $n = 97$ (%)	OR (95% CI)	*p* Value	Power
IL18 -607										
C	146 (0.55)	117 (0.48)	1.34 (0.95–1.90)	0.0975	0.32	21 (0.42)	96 (0.49)	0.74 (0.39–1.39)	0.3449	0.11
A	118 (0.45)	127 (0.52)				29 (0.58)	98 (0.51)			
C/C	33 (0.25)	32 (0.26)				4 (0.16)	28 (0.29)			
C/A	80 (0.61)	53 (0.43)	–	0.0043		13 (0.52)	40 (0.41)	–	0.4038	
A/A	19 (0.14)	37 (0.30)				8 (0.32)	29 (0.30)			
IL18 -137										
G	178 (0.67)	182 (0.75)	0.71 (0.48–1.04)	0.0757	0.49	33 (0.66)	149 (0.77)	0.59 (0.30–1.15)	0.1176	0.32
C	86 (0.33)	62 (0.25)				17 (0.34)	45 (0.23)			
G/G	55 (0.42)	66 (0.54)				11 (0.44)	55 (0.57)			
G/C	68 (0.52)	50 (0.41)	–	0.1382		11 (0.44)	39 (0.40)	–	0.1445	
C/C	9 (0.07)	6 (0.05)				3 (0.12)	3 (0.03)			
TNFA -308										
G	197 (0.75)	148 (0.61)	1.91 (1.31–2.78)	0.0008		32 (0.64)	116 (0.60)	1.20 (0.63–2.28)	0.5872	
A	67 (0.25)	96 (0.39)				18 (0.36)	78 (0.40)			
G/G	70 (0.53)	37 (0.30)				8 (0.32)	29 (0.30)			
G/A	57 (0.43)	74 (0.61)	–	0.0008		16 (0.64)	58 (0.60)	–	0.6174	
A/A	5 (0.04)	11 (0.09)				1 (0.04)	10 (0.10)			

HPV, human papillomavirus; SNP, single nucleotide polymorphism; HC, healthy controls; HPV+, persons infected by human papillomavirus; OR, odds ratio; CI, confidence interval; LSIL, Low-grade Squamous Intraepithelial Lesion; HSIL, High-grade Squamous Intraepithelial Lesion.

HPV. In a recent study using a transgenic mouse model, Gosmann et al. (2014) reported the activation of an immunosuppressive environment in hyperplastic transgenic mice skin, driven by IL-18 that stimulates IFN-γ production. These results could support the findings of Sobti et al. (2008), which describe an association between IL-18 promoter polymorphisms and the risk of developing HPV-associated cervical cancer in an Indian population. On the other hand, Yang et al. (2013) reported that *IL18* polymorphisms (namely -1297 T/C, -607 C/A, -380 C/G, -137 G/C and +105 A/C, so including those analysed in our study) were not associated with susceptibility to cervical squamous cell carcinoma in HPV infected women from Taiwan.

The association between *IL18* -607 AA genotype, reported to be responsible for lower IL18 levels, with protection against HPV infection, does not fit with the considerations made above, since a decreased IL18 production should be related to a defective activation of the immune system against HPV, thus not conferring protection but increased risk for infection. However, our genetic results have been obtained on a Brazilian population, ethnically different from those analysed by Sobti et al. (2008). This discrepancy once more highlights the importance of replica studies which are needed to double check the results obtained and does suggest that each genetic association study should be completed by evaluation of protein levels (in our case TNF-α and IL18) to clarify the genotype–phenotype correlation.

We also found an association between smoking and HSIL presence, as observed by some authors, but the results are controversial (Fernandes et al., 2009; Lamb et al., 2013; Matsumoto et al., 2010; Trimble et al., 2005; Wilkerson & Prosser, 2010). Other authors point to a beneficial effect of smoking cessation in relation to the development of cervical lesions. In a longitudinal study, 82 women with low-grade lesions were encouraged to quit smoking. After 6 months of follow-up, lesions in 50% of those who stopped smoking disappeared, while higher-grade lesions appeared in those who did not (Szarewski et al., 1996). In the Manchester cohort, HPV positive women smoking for 10 years or more and those who smoked more than 10 cigarettes per day had a higher risk of high-grade lesions than those who never smoked (Deacon et al., 2000).

Although alcohol consumption may also be associated with the risk of developing low-grade lesions in HR-HPV-positive women (Min et al., 2013), we did not find similar results. However, we did not analyse the quantity of alcohol consumption per day, only whether the patient did or did not consume alcohol. Thus, methodological differences could explain this discordance.

Conclusions

Our study is suffering from some strong limitations represented by: the modest number of subjects analysed; the low values of statistical power ($p = 0.49$ and 0.32, when acceptable Power is >0.80), quite weak to support our association findings; and the lack of quantitative assessment of the effect of the genetic polymorphisms on the cytokine expression levels in serum. So, being aware of these important limitations and considering the discordant results with the literature, we can conclude that, in our Brazilian population, ***TNFA*** -308 GG genotype and G allele were significantly associated with increased risk of HPV infection, while *IL18* -607 AA genotype conferred protection against HPV-infection. However, these two polymorphisms were not associated with progression to HPV-related cervical lesions, so we should be extremely cautious in the pathogenic interpretation of our findings, since TNF-α and IL18 were also supposed to affect HPV spread and persistence.

Finally, among the host behavioural variables, a positive association was observed between smoking and HSIL. We performed a logistic regression analysis in order to evaluate the potential risk factors for the development of high-grade HPV lesions (HSIL). After controlling for HPV infection status (single infection or co-infection), cytokine genotypes and alcohol consumption, only smoking was associated with higher risk for HSIL, thus re-inforcing the notion that smoking is closely linked to carcinogenesis.

In light of our results and those reported in the literature, we do highlight the strong need for further replica studies before drawing any consistent conclusion about the role of *TNFA* and *IL18* genetic variants as markers for HPV infection and persistence.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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ANEXO C – ARTIGO PUBLICADO

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Influence of *IL-6*, *IL-8*, and *TGF-β1* gene polymorphisms on the risk of human papillomavirus-infection in women from Pernambuco, Brazil

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Human papillomavirus (HPV) infections are strongly associated with the development of cervical intraepithelial neoplasias and invasive cervical cancer. Polymorphisms in cytokine-encoding genes and behavioural cofactors could play an important role in protecting an individual against viral infections and cancer. Here, we investigated whether IL-6 *-174 G>C,* IL-8 *+396 G>T, and* TGF-β1 *+869 G>C and +915 G>C polymorphisms were associated with susceptibility to HPV infection in women from north-east (Pernambuco) Brazil. We analysed 108 healthy uninfected women (HC) and 108 HPV-positive women with cervical lesions. Genetic polymorphisms were assessed using Sanger sequencing and polymerase chain reaction-restriction fragment length polymorphism. Comparison of the distribution of the genotypic and allelic frequencies of the IL-18 +396 T>G polymorphism between HPV infected woman an uninfected controls showed that the GG genotype and G allele were both more frequent in the HC group, and were associated with protection from HPV infection (p = 0.0015; OR = 0.29 CI95% = 0.13-0.61; p = 0.0005; OR = 0.45 CI95% 0.29-0.7, respectively). Individuals from the control group could have previously had HPV infection that was spontaneously eliminated; however, it was undetectable at the time of sample collection. Based on our findings, we hypothesize that the* IL-8 *+396 G>T polymorphism could interfere with susceptibility to HPV infection, by modulating the ability of immune system to fight the virus.*

Key words: polymorphisms - cytokine - host genome - HPV

Cervical cancer represents a significant public health problem causing a great impact worldwide; every year approximately 528,000 new cases and 266,000 deaths (7.5% of all female cancers) are expected (IARC 2012). In Brazil, cervical cancer is the second most common type of cancer among women. Approximately 16,340 new cases are expected for 2016, representing 15.85 cases per 100,000 individuals (INCA 2016). Infections with oncogenic types of the human papillomavirus (HPV) are responsible for most cases of cervical cancer and precancerous intraepithelial lesions (Muñoz et al. 2003).

Although the incidence of genital HPV infections is high, most of these infections are transient and do not lead to cervical intraepithelial neoplasia (CIN) or cancer, suggesting that other factors such as the immune system as well as the host genetic background could influence disease risk (Wu & Levine 1994).

HPV infection promotes immune cell migration to the dermis. In the squamous epidermis, macrophages, Langerhans cells (LC), T lymphocytes, dendritic cells (DC), natural killer cells (NK), and B-lymphocytes play important roles in the immune response to infection. HPV infection could induce the immune system to become more tolerant to the infection, thereby creating a microenvironment susceptible to further infection, facilitating CIN progression (Song et al. 2015).

These local immune responses seem to play an important role in the natural history of HPV infection of the uterine cervix. Cytokines are important regulators of HPV transcription due to their important role in the defence against HPV infection, through modulating viral replication (zur Hausen 2002). Polymorphisms in genes related to immunity have been reported to influence susceptibility to several diseases including viral infections. Therefore, these polymorphisms should also be considered when analysing the genetic susceptibility to HPV infection as well as cervical cancer (Wang & Hildesheim 2003).

The discovery of a TH17 variant has improved the understanding of inflammatory processes. TH17 cells are linked to chronic neutrophilic inflammation and can be induced to differentiate by transforming growth factor β1 (TGF-β1) and interleukin-6 (IL-6). Therefore, TGF-β1 and IL-6 can also induce the differentiation of TH9 and TH22 cells, respectively (both linked to tissue inflammation) (Akdis et al. 2011).

The IL-6 gene, located on the short arm of human chromosome 7 (7p21), presents several single nucleotide polymorphisms (SNPs), one of which is localised in the

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promoter region (-174G>C, rs1800795), and is associated with variations in *IL-6* expression and on serum protein levels (Fishman et al. 1998). This SNP correlates with poor prognosis in gastric cancer (de Vita et al. 2001) and prostate cancer (Nakashima et al. 2000). Moreover, IL-6 seems to be involved in cervical cancer progression and metastasis (Kinoshita et al. 1999, de Vita et al. 2001).

TGF-β1, a fundamental molecule involved in the homeostasis between cellular growth and apoptosis, is encoded by the *TGF-β1* gene located on the long arm of human chromosome 19 (19q13). The +869 T>C (rs1982073) SNP is located at codon 10 of exon 1 and results in a leucine-to-proline substitution, whereas +915 G>C (rs1800471) is located at codon 25 and results in an arginine-to-proline change. The C variant allele of codon 10, as well as the wild-type G allele of codon 25, is associated with increased production of TGF-β1 (Dunning et al. 2003). Furthermore, circulating levels of TGF-β1 have been associated with several diseases, including cancer (Elliott & Blobe 2005).

Interleukin-8 (IL-8), the first chemokine discovered, has pro-inflammatory activity, and is produced as part of immune or acute inflammatory reactions, as well as during chronic inflammation; this cytokine functions to attract and activate neutrophils in inflammatory regions (Campa et al. 2005). The *IL-8* gene is located on the long arm of chromosome 4 (4q12-q21) and several promoter, intron, and 3′UTR SNPs have been identified (Hull et al. 2001). The *IL-8* +396 G>T (rs2227307) polymorphism has been associated with respiratory diseases caused by viruses (Hull et al. 2001) and gastric cardiac adenocarcinoma (Savage et al. 2004).

Since few studies have been performed correlating SNPs in *IL-6*, *IL-8*, and *TGF-β1* genes with the risk of HPV infection, we evaluated whether *IL-6* (-174 G>C), *IL-8* (+396 G>T), and *TGF-β1* (+869 T>C and +915 G/C) functional genetic variants were related to HPV susceptibility, by studying HPV infected and uninfected women from Pernambuco, Brazil.

SUBJECTS, MATERIALS AND METHODS

Patients - A hospital-based cross-sectional study was performed aimed at analysing *IL-6, IL-8,* and *TGFβ1* gene polymorphisms in HPV-infected women with cervical lesions and invasive cervical cancer (ICC). One hundred and eight women aged 17-68 years, with a mean age of 33.9 ± 10.1 years, presenting with either low-grade squamous intraepithelial lesion (LSIL) or high-grade squamous intraepithelial lesion (HSIL), were selected from the outpatient clinics of the Lower Genital Tract Pathology Clinic at the Women's Healthcare Centre of the Prof Fernando Figueira Institute of Integrated Medicine, (Recife, Pernambuco, Northeast Brazil). Patients were selected by spontaneous demand from January 2009 to December 2010. Patients were included in the study if there was no discrepancy between cytological abnormality and histological diagnosis made at the first visit. All patients were initially assessed by colposcopy and subsequently cervical smears were collected. Histological diagnosis was made according to the Associação Brasileira de Genitoscopia (ABG 2002). Subjects were evaluated for clinical features of other sexually transmitted infections (STIs) based on history and examination. Patients who were previously submitted to radiotherapy or chemotherapy for ICC were excluded. Patients were also stratified according to age, parity, number of partners, smoking, and alcohol consumption.

One hundred and eight unrelated women from Pernambuco, enrolled at the Women's Healthcare Centre, the same as that of HPV infected patients, aged between 14-66 years (mean age 37.2 ± 10 years), with no history of lesions or neoplastic disease as evaluated by the physician, and testing negative for HPV infection, were used as controls.

Informed written consent was obtained from the women, and the women were informed of the background of the study, risks and benefits, and voluntary nature of participation (CEP/CCS/UFPE N° 355/08).

Clinical samples - Cervical smears were obtained using cytobrushes. Each cytobrush was packed in a TE buffer solution (10 mM Tris-HCl and 1 mM EDTA, pH 8.0) and maintained at -20°C until analysis.

DNA extraction - Genomic DNA extraction was performed from 300 µL of cervical smear, from each study subject, using the Wizard Genomic DNA Purification kit (Promega, Madison, WI, USA) following the manufacturer's instructions. The analyses were executed in Laboratory of Genetic, Biochemistry and DNA Sequencing (LGBS) of Rural Federal University of Pernambuco.

HPV DNA detection - Human papillomavirus DNA was detected from DNA extracted as previously described using two polymerase chain reaction (PCR) steps. The first was with MY09/11 external primers (MY09 5′-CGTCCMARRGGAWACTGATC-3′ and MY11 5′-GCMCAGGGWCATAAYAATGG-3′) and the second was with GP05+/06+ (GP5+ 5′-TTTGTTACTGTGGTAGATACTAC-3′ and GP6+ 5′-GAAAAATAAACTGTAAATCATATTC-3′) as internal ones. These two primers pairs are most widely used for the detection of genital HPVs. A negative control containing only digestion buffer was included for every five samples to prevent and detect carry-over between samples.

IL-6, IL-8, and TGF-β1 SNPs genotyping - *IL-6* and *TGF-β1* polymorphisms were amplified from the same DNA utilised for HPV detection, using specific primers (*IL-6* - 5′-TTGTCAAGACATGCCAAAGTG-3′ and 5′-TCAGACATCTCCAGTCCTATA-3′ and *TGF-β1* - 5′-TTCCCTCGAGGCCCTCCTA-3′ and 5′-GCCGCAGCTTGGACAGGATC-3′) which flanked the polymorphisms. PCR was performed following standard protocols from the literature. Briefly, after amplification, the amplicons were submitted to a sequencing reaction using the DyEnamic ET Dye Terminator Cycle sequencing kit (GE Healthcare) according to manufacturer's recommendations and were sequenced using a MegaBACE 1000 DNA Sequencer.

IL-8 was amplified from the same DNA utilised for HPV detection using specific primers (5 ′-TAAAGGTTTGATCAATATAGA-3′ and 5′-CTTCCTTCTAATTCCAATATG-3′) according to literature, and the genotypes were identified through restriction fragment

analysis polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) using the *ScrFI* restriction enzyme. Thirty per cent of the samples were sequenced to confirm the results.

Statistical analysis - Univariate statistical analysis was performed using BioEstat 5.0 software. The study was cross-sectional, with independent samples consisting of nominal data (genotypes). The influence of polymorphism on the risk for development of (pre) neoplastic cervical disease was estimated by an odds ratio (OR) and a 95% confidence interval (CI). Allele frequencies were estimated by direct counting. Comparison between genotypic frequencies of patients and control groups was performed using a $\chi 2$ test; a Fisher's exact test was used to compare the allele frequencies in contingency tables. The OR and their respective 95% CI values were determined. All p values ≤ 0.05 were considered statistically significant.

RESULTS

Selected characteristics, including age, age of first sexual coitus, parity, smoking status, alcohol consumption, and oral contraceptive use of 108 HPV infected women with cervical lesions are summarised in Table I; 44 women (40.74%) were over 35 years of age; 73 (67.6%) were younger than 18 at the time of first sexual intercourse; 23 (21.3%) had a parity of five or more; 64 (59.3%) women reported regular use of alcohol; 41 (37.9%) women were smokers, and 55 (50.9%) women reported the use of oral contraceptives. There was no significant difference between women with HSIL and LSIL in terms of age of first sexual intercourse (p = 0.3787), parity (p = 0.5637), smoking (p = 0.8130), alcohol consumption (p = 0.9781), and oral contraception use (p = 0.9247). HSIL and LSIL were more frequent in subjects aged ≥ 35 years than in subjects < 35 years of age (p = 0.0124) (Table I).

Table II shows the distribution of genotypes and allele frequencies of *IL-6* (-174 G>C), *IL-8* (+396 T>G), and *TGF-β1* (+869 G>C) and (+915 G>C) polymorphisms in HPV infected women and healthy uninfected controls. *IL6* and *TGF-β1* (+869 T>C) and (+915 G>C) polymorphism frequencies were in Hardy-Weinberg equilibrium in HPV infected women and uninfected controls, whereas the *IL-8* +396 T>G polymorphism showed frequencies not in agreement with the Hardy-Weinberg equilibrium in the two groups of subjects analysed.

IL-6 -174 G>C and *TGF-β1* +869 T>C and +915 G>C polymorphism alleles and genotype frequencies were not statistically different between HPV infected women and healthy uninfected controls. Comparison of the *IL-8* +396 T>G polymorphism allele and genotype frequencies between HPV infected woman and uninfected controls showed a statistically significant increase in the frequency of the GG genotype in uninfected subjects compared to HPV infected women, suggesting an association between this allele and protection from HPV infection (p = 0.0015; OR = 0.29 CI95% 0.13-0.61). In addition, the prevalence of the G allele was more frequent in HCs than in HPV infected women, thus, suggesting an association between this allele and protection from HPV infection (p =0.0005; OR = 0.45 CI95% 0.29-0.70).

TABLE I

Associations of clinical characteristics between human papillomavirus infected women with high-grade squamous intraepithelial lesions (HSIL) and those with low-grade squamous intraepithelial lesions (LSIL)

	LSIL	HSIL	OR	95%CI	P
Age					
< 35	18 (85.7%)	46 (52.9%)		1	
≥ 35	3 (15.3%)	41 (47.1%)	5.35	1.47 - 19.48	0.0124*
AFSI					
> 18 years old	9 (42.8%)	26 (29.9%)		1	
< 18 years old	12 (57.2%)	61 (70.1%)	1.76	0.66 - 4.68	0.3787
Parity					
< 5 births	18 (85.7%)	67 (77%)	1		
≥ 5 births	3 (15.3%)	20 (23%)	1.79	0.48 - 6.70	0.5637
Smoking					
No	14 (66.7%)	53 (60.9%)		1	
Yes	7 (33.3%)	34 (39.1%)	1.28	0.47 - 3.50	0.8130
Alcohol consumption					
No	8 (38.1%)	36 (41.4%)		1	
Yes	13 (61.9%)	51 (58.6%)	0.87	0.33 - 2.32	0.9781
OCP use					
No	11 (52.4%)	42 (48.3%)		1	
Yes	10 (47.6%)	45 (51.7%)	1.18	0.45 - 3.06	0.9247

AFSI: age of first sexual intercourse; OCP use: oral contraceptive use; OR: odds ratio; 95%CI: 95% confidence interval; P: p-value; *: statistically significant.

TABLE II

Allele and genotype frequencies of *IL-6* (-174 G>C), *IL-8* (+396 G>T), and *TGF-β1* (+869 G>C) and (+915 G>C) polymorphisms among human papillomavirus (HPV) positive women and healthy uninfected controls from Pernambuco state (Northeast Brazil)

	HPV infected women	Healthy uninfected controls	OR (CI 95%)	P value
IL6				
Allele	n = 216 (Freq)	n = 216 (Freq)		
G	172 (0,8)	163 (0,75)	Ref	
C	44 (0,2)	53 (0,25)	0,79 (0,5 - 1,24)	0,3563
Genotype	n = 108 (Freq)	n = 108 (Freq)		
G/G	67 (0,62)	64 (0,59)	Ref	0,2027
G/C	38 (0,35)	35 (0,32)	1,04 (0,58 - 1,84)	0,9829
C/C	3 (0,03)	9 (0,08)	0,32 (0,08 - 1,23)	0,152
IL8				
Allele	n = 216 (Freq)	n = 216 (Freq)		
T	174 (0,81)	141 (0,65)	Ref	
G	42 (0,19)	75 (0,35)	0,45 (0,29 - 0,7)	0,0005*
Genotype	n = 108 (Freq)	n = 108 (Freq)		
T/T	77 (0,71)	66 (0,61)	Ref	0,0003*
T/G	20 (0,19)	9 (0,08)	1,9 (0,81 - 4,47)	0,1964
G/G	11 (0,1)	33 (0,31)	0,29 (0,13 - 0,61)	0,0015*
TGF-β1+869T/C				
Allele	n = 216 (Freq)	n = 216 (Freq)		
T	131 (0,61)	131 (0,61)	Ref	
C	85 (0,39)	85 (0,39)	1 (0,68 - 1,47)	0,9215
Genotype	n = 108 (Freq)	n = 108 (Freq)		
T/T	40 (0,37)	37 (0,34)	Ref	0,6905
T/C	51 (0,47)	57 (0,53)	0,83 (0,46 - 1,49)	0,628
C/C	17 (0,16)	14 (0,13)	1,12 (0,49 - 2,59)	0,9528
TGF-β1+915G/C				
Allele	n = 216 (Freq)	n = 216 (Freq)		
G	210 (0,97)	207 (0,96)	Ref	
C	6 (0,03)	9 (0,04)	0,66 (0,23 - 1,88)	0,5992
Genotype	n =n108 (Freq)	n = 108 (Freq)		
G/G	102 (0,94)	99 (0,92)	Ref	
G/C	6 (0,06)	9 (0,08)	0,65 (0,22 - 1,89)	0,5924
C/C	0 (0)	0 (0)		

CI95%: 95% confidence interval; OR: Odds ratio; Ref: reference Allele; *: statistically significant.

In addition, with the aim of comparing the allele frequencies of the SNPs observed in our study with those of the population from Pernambuco, we inferred the allele frequencies (indicated as f in the equation below; p indicates proportion) for each polymorphism based on the equation presented by Suárez-Kurtz et al. (2014):

$$f\_Brazil = (f_{Portugal} * p_{European}) + (f_{(Angola+Mozambique)} * p_{\_African}) + (f_{Guarani} * p_{\_Amerindian})$$

Essentially, this equation calculates a weighted average of the allele frequency observed in the parental populations of Brazil, considering the proportion of each ancestry found in the Brazilian population. Since we had no data regarding the populations described in the equation, we used the findings of some related populations with available data present in the 1000 Genomes Project database (http://browser.1000genomes.org) as proxies.

For each polymorphism, we obtained the allele frequencies from European Iberian (IBS), African Yoruba (YRI), and Peruvian South American (PEL) genome databases and combined the frequencies considering the admixture of the Pernambuco population, comprising genomes resulting from European (59.7%), African (23%), and Amerindian (17.3%) ancestries, as described in Coelho et al. (2015).

The predicted admixed frequencies for each polymorphism were as follows: 0.22 for the *IL-6* (-174 G>C) polymorphism; 0.41 for the *IL-8* (+396 T>G) polymorphism; 0.46 for *TGF-β1* (+869 T>C); 0.03 for the *TGF-β1* (+915 G>C) SNP. Finally, no significant differences in the dis-

tribution of *IL-6* (-174 G>C), *IL-8* (+396 T>G), *TGF-β1* (+869 T>C), and (+915 G>C) polymorphisms were observed between LSIL and HSIL groups (Table III) in HPV infected women.

DISCUSSION

Since HPV infection is a multifactorial process, depending on environmental and host genomic factors, with particular focus on immunoregulatory genes, we analysed the possible impact of genetic variants, namely *IL-6* (-174 G>C), *IL-8* (+396 T>G), and *TGF-β1* (+869 T>C) and (+915 G>C) (cytokines encoding genes), on the susceptibility to HPV infection in women (HPV infected and uninfected) from Pernambuco (Brazil).

We did not find any significant association between the *IL-6* +174 G>C polymorphism and HPV infected women or uninfected controls, or between HSIL and LSIL subgroups; we also did not find any relationship between *TGF-β1* +869 T>C and +915 G>C polymorphisms and the risk of HPV infection or the development of LSIL or HSIL. *IL-6* and *TGF-β1* polymorphisms were not associated with the development of cervical cancer in a population from Zimbabwe, Africa (Stanczuk et al. 2002), and in a population from Shaaxi, China (Wang et al. 2011). In Brazil, Fernandes et al. (2008) did not find any correlation between *TGF-β1* +869 G>C and +915 G>C polymorphisms and the risk for HPV-related cervical lesions in women from São Paulo. Marangon et al. (2013) reported similar findings in HPV infected women from Paraná, Brazil.

Analysis of the *IL-8* +396 T>G polymorphism revealed an increased frequency of the TT genotype in HPV infected women, whereas the GG genotype and the G allele were both more frequent in healthy uninfected women, and were thus associated with protection from HPV infection.

It is worth noting that both HPV and HC groups were not in Hardy-Weinberg Equilibrium for the *IL-8* +396 T>G SNP due to an excess of homozygosity when compared to the expected genotype frequencies predicted for the general population of Recife. Furthermore, the minor allele frequency of this polymorphism in both HPV (0.19) and HCs (0.35) was reduced in comparison to that predicted by ancestry estimates (0.41). Therefore, more studies are needed to investigate the lower than expected heterozygosity in this population.

IL-8, a chemokine, acts by attracting granulocytes to sites of inflammation, resulting in neutrophil-mediated viral clearance (Knall et al. 1997). IL-8 expression is primarily regulated by an activator protein and/or nuclear factor-κB-mediated transcriptional activity, which is a critical regulator of the immediate early pathogen response that acts in response to bacterial and viral infections (Brat et al. 2005).

IL-8 has been hypothesized to play an important role in ovarian cancer (Kassim et al. 2004). Increased IL-8 serum levels were related to HPV persistence (Baker et al. 2011); IL-8 has been also reported to be involved in the epithelial-mesenchymal transition and the tumour microenvironment (Palena et al. 2012). Some viruses such as Herpes Simplex Virus (HSV), Epstein-Barr Virus (EBV), Human Immunodeficiency Virus (HIV) and Human T Lymphotropic Virus (HTLV-1) have been shown to induce IL-8 expression through the Nuclear Factor-κB (NF-κB) pathway (Mogensen & Paludan 2001).

TABLE III

Association of *IL-6* (-174 G>C), *IL-8* (+396 T>G), and *TGF-β1* (+869 G>C) and (+915 G>C) polymorphisms among human papillomavirus (HPV) positive women with high-grade squamous intraepithelial lesions (HSIL) and those with low-grade squamous intraepithelial lesions (LSIL)

	LSIL	HSIL	OR	95%CI	P		LSIL	HSIL	OR	95%CI	P
IL-6						TGF-β1+869 T/C					
GG	13 (61.9%)	54 (62.1%)		1		TT	11 (52.4%)	29 (33.3%)		1	
GC	7 (33.3%)	31 (35.6%)	1.07	0.38 - 2.95	0.8923	TC	8 (38.1%)	43 (49.5%)	2.04	0.73 - 5.68	0.2343
CC	1 (4.8%)	2 (2.3%)	0.48		0.8827	CC	2 (9.5%)	15 (17.2%)	2.84	0.56 - 14.53	0.3420
G	33 (78.6%)	139 (79.9%)	1			T	30 (71.4%)	101 (58.1%)		1	
C	9 (21.4%)	35 (20.1%)	0.92	0.40 - 2.11	0.9811	C	12 (28.6%)	73 (41.9%)	1.81	0.87 - 3.76	0.1564
IL-8						TGF-β1+915 G/C					
GG	1 (4.8%)	10 (11.5%)		1		GG	20 (95.2%)	82 (94.2%)		1	
GT	8 (38.1%)	12 (13.8%)	0.15	0.02 - 1.41	0.1614	GC	1 (4.8%)	5 (5.8%)	1.22	0.13 - 11.03	0.7235
TT	12 (57.1%)	65 (74.7%)	0.54	0.06 - 4.63	0.9096	CC	0 (0%)	0 (0%)		nd	
G	10 (23.8%)	32 (18.4%)	1			G	41 (97.6%)	169 (97.1%)		1	
T	32 (76.2%)	142 (81.6%)	1.39	0.62 - 3.11	0.5625	C	1 (2.4%)	5 (2.9%)	1.21	0.14 - 10.67	0.7273

OR: odds ratio; 95%CI: 95% confidence interval; P: p-value.

Differential expression of IL-8 could be associated with the presence of functional polymorphisms such as the *IL-8* +396 G/T. The *IL-8* +396 GG genotype is known to yield higher levels of IL-8 and has been linked to the development of ovarian carcinoma (Wang et al. 2012). Therefore, we hypothesize that individual carriers of the *IL-8* +396 GG genotype, associated with high levels of IL-8, are better able to control HPV infection through the attraction of granulocytes to the sites of infection. Consequently, this would results in successful neutrophil-mediated viral clearance. Nevertheless, this is a hypothesis that needs to be verified by analysing IL8 serum levels, was not possible to analyse in this study. Moreover, the *IL-8* +396 T/G polymorphism allele and genotype frequencies assessed in HPV infected and uninfected women were not in Hardy-Weinberg equilibrium and were different from those inferred for the Pernambuco population. Therefore, our findings should be considered with caution and thus need to be replicated in at least one other north-east Brazilian population.

We are also aware that the main limitation of this study is the relatively small cohort enrolled; furthermore, HPV high-risk and low-risk genotyping data were not available to us; therefore, it was impossible to evaluate the impact of HPV genotypes on the susceptibility to cervical lesions as well as their relationship with cytokine-encoding gene polymorphisms.

In conclusion, taking into account the fact that individuals from the control group could have been previously infected by HPV that was spontaneously eliminated, and based on our findings, we hypothesize that the *IL-8* +396 G allele and GG genotypes could play a role in the risk of HPV infection in our study population. Specifically, carriers of these genetic variants, responsible for higher IL-8 production, should be able to react better to HPV infection. This work will prompt future genetic studies regarding the molecular pathogenesis of HPV infection and similar analyses should be performed to determine whether this polymorphism could serve as a prognostic risk factor for HPV infection.

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